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(71) Applicants *(for all designated States except US)*: GENETICS INSTITUTE, INC. [US/US]; 87 CambridgePark Drive, Cambridge, MA 02140 (US). CBR LABORATORIES, INC. [US/US]; 800 Huntington Avenue, Boston, MA 02115 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MANJUNATH, Narasimhaswamy [US/US]; 28 Weld Street, Roslindale, MA 02131 (US). HANS VON ANDRIAN, Ulrich [US/US]; 50 Lancaster Terrace #3, Brookline, MA 02446 (US).

(74) Agent: MANDRAGOURAS, Amy, E.; Lahive & Cockfield, LLP, 28 State Street, Boston, MA 02109 (US).

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WO 00/25808 A1

(54) Title: INHIBITION OF DIFFERENTIATION OF CYTOTOXIC T-CELLS BY P-SELECTIN LIGAND (PSGL) ANTAGONISTS

(57) Abstract: Methods are disclosed for inhibiting the differentiation of an activated T-cell into a cytotoxic lymphocyte in a mammalian subject, comprising administering to a subject a therapeutically effective amount of a PSGL antagonist.

INHIBITION OF DIFFERENTIATION OF CYTOTOXIC T-CELLS BY P-SELECTIN LIGAND (PSGL) ANTAGONISTS

Background of the Invention

P-selectin is a cell adhesion molecule expressed, among other places, on vascular endothelium. Interaction of P-selectin with its ligand, PSGL (also known as "PSGL-1", which is expressed, among other places, on neutrophils), causes cells circulating in the vasculature which express PSGL to attach to the endothelium, where other adhesion molecules mediate extravasation into the surrounding tissues. Thus, the P-selectin/PSGL interaction has been a well-documented step in the development of inflammatory and immune responses.

PSGL has been cloned and well-characterized as described in International Application No. WO98/08949 (which is incorporated herein by reference). Such application discloses polynucleotides encoding various forms of PSGL, including numerous functional soluble forms of PSGL. Thus, PSGL is a well-characterized molecule, the soluble forms of which are particularly amenable to administration as therapeutics.

Therefore, it would be desirable to determine whether PSGL is involved in other cellular interactions for which forms of PSGL or other PSGL antagonists could serve as inhibitors.

25. The method of claim 24 wherein the sulfated, glycosylated peptides are expressed in the eukaryotic cells expressing an α 1-3 fucosyltransferase, an α 1-4 fucosyltransferase, or an α 1/3-1-4 fucosyltransferase (FTIII) and a core 2 β1-6-N-acetylglucosaminyltransferase.

26. The method of claim 24 wherein the cells are mammalian and the glycosyltransferases are human.

27. The method of claim 24 wherein the peptides are selected from the group consisting of peptides corresponding to Sequence ID No. 1 from 42 to 57, 42 to 67, 42 to 69, 42 to 70, 42 to 73, 42 to 77, 42 to 80, 42 to 81, 42 to 82, 42 to 90, 42 to 91, 42 to 100, 42 to 103, 42 to 104, and these peptides having conservative substitutions in these peptides which do not alter the sulfated tyrosines or attachment of carbohydrate in a way decreasing binding of the peptide to P-selectin.

Summary of the Invention

Applicants have for the first time determined that soluble PSGL or antibodies directed to PSGL will inhibit the differentiation of activated proliferating T-cells into cytotoxic lymphocytes. Thus, soluble PSGL, PSGL antibodies and other PSGL antagonists will inhibit such differentiation and the attendant immune and inflammatory responses resulting therefrom. As a result, these antagonists can be used to treat diseases and other conditions which result from undesirable or over-aggressive immune and inflammatory responses, such as, for example, in allergic reactions and autoimmune conditions.

The present invention provides a method of inhibiting the differentiation of an activated T-cell into a cytotoxic lymphocyte in a mammalian subject, said method comprising administering to said subject a therapeutically effective amount of a PSGL antagonist.

Other embodiments provide for a method of treating or ameliorating an autoimmune condition, said method comprising administering to said subject a therapeutically effective amount of a PSGL antagonist.

Yet other embodiments provide for a method of treating or ameliorating an allergic reaction, said method comprising administering to said subject a therapeutically effective amount of a PSGL antagonist.

Other embodiments provide a method of treating or ameliorating asthma, said method comprising administering to said subject a therapeutically effective amount of a PSGL antagonist.

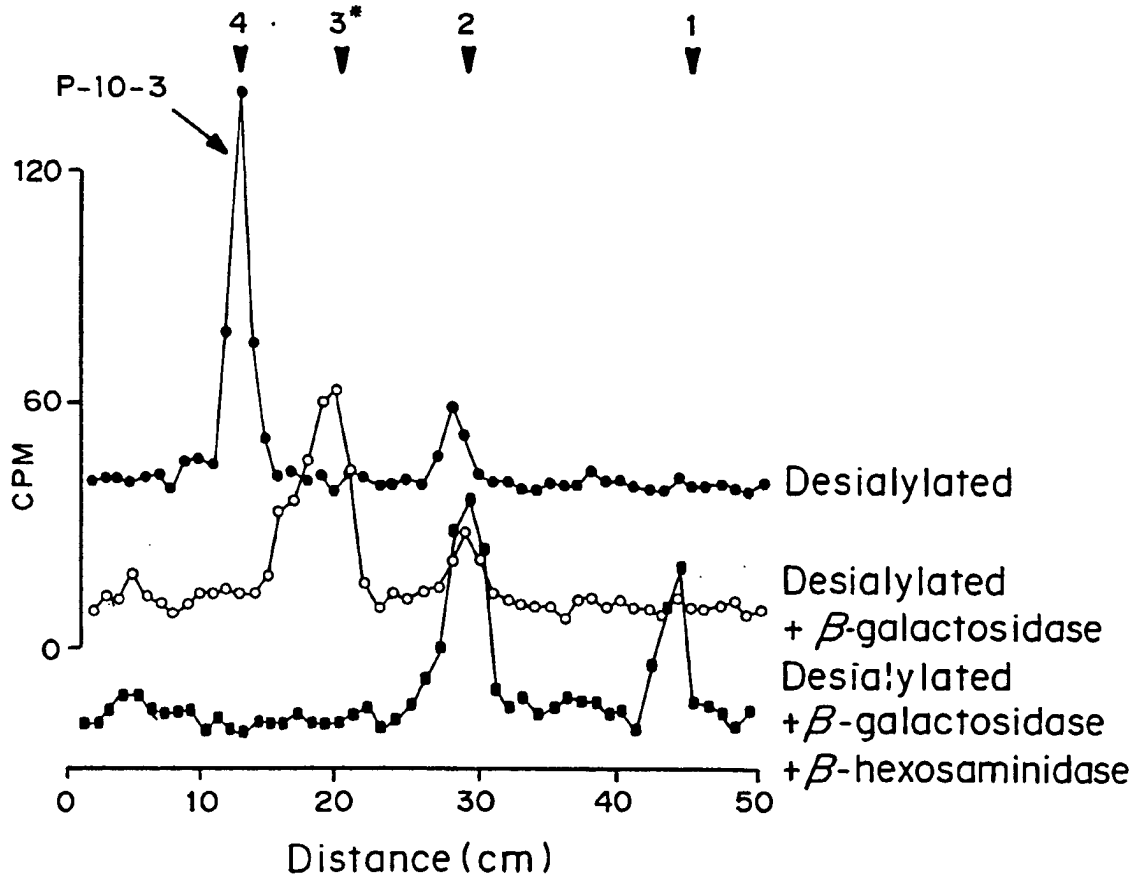
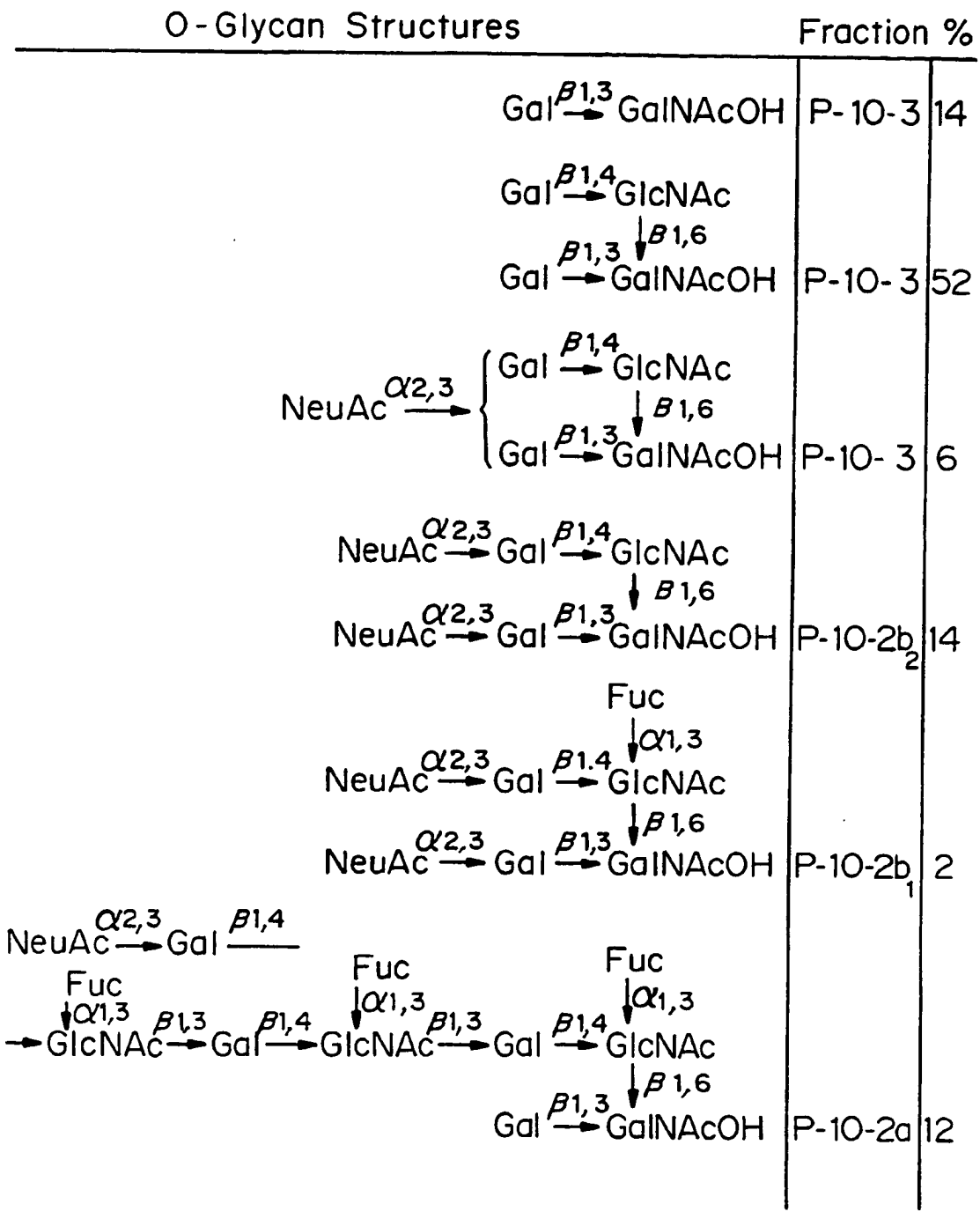

4
3*
2
1
P-10-3
120
60
0
CPM
Desialylated
Desialylated
+ β-galactosidase
Desialylated
+β-galactosidase
+β-hexosaminidase
0
10
20
30
40
50
Distance (cm)

FIG. 6

FIG. 7


O-Glycan Structures
Fraction %
Gal β1,3 GalNAcOH
P-10-3
14
Gal β1,4 GlcNAc
β1,6
Gal β1,3 GalNAcOH
P-10-3
52
NeuAc α2,3
Gal β1,4 GlcNAc
β1,6
Gal β1,3 GalNAcOH
P-10-3
6
NeuAc α2,3 Gal β1,4 GlcNAc
β1,6
NeuAc α2,3 Gal β1,3 GalNAcOH
P-10-2b2
14
Fuc
α1,3
NeuAc α2,3 Gal β1.4 GlcNAc
β1,6
NeuAc α2,3 Gal β1,3 GalNAcOH
P-10-2b1
2
NeuAc α2,3 Gal β1,4
Fuc
α1,3
Fuc
α1,3
Fuc
α1,3
GlcNAc β1,3 Gal β1,4 GlcNAc β1,3 Gal β1,4 GlcNAc
β1,6
Gal β1,3 GalNAcOH
P-10-2a
12

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A61K 31/70, 38/14, 38/17

A3

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(63) Related by Continuation
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(71) Applicant *(for all designated States except US)*: BOARD OF REGENTS OF THE UNIVERSITY OF OKLAHOMA [US/US]; 600 Parrington Oval, Norman, OK 73109 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MCEVER, Rodger, P. [US/US]; 1716 Guilford Lane, Oklahoma City, OK 73120 (US). CUMMINGS, Richard, D. [US/US]; 5215 Santa Fe, Edmond, OK 73034 (US). MOORE, Kevin, L. [US/US]; 612 N.W. 42nd Street, Oklahoma City, OK 73118 (US).

(74) Agent: PABST, Patrea, L.; Arnall Golden & Gregory, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GA 30309-3450 (US).

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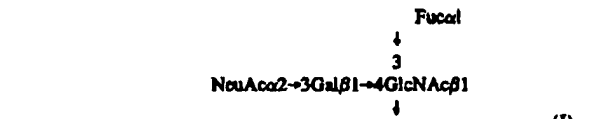
24 July 1997 (24.07.97)

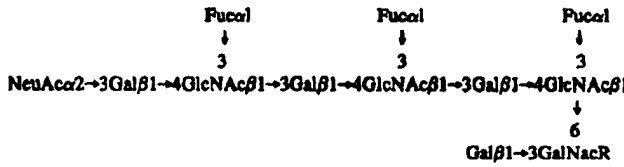
(54) Title: PEPTIDE AND O-GLYCAN INHIBITORS OF SELECTIN MEDIATED INFLAMMATION

(57) Abstract

Tyrosine sulfate on PSGL-1, particularly at least one of residues (46, 48) and (51), functions in conjunction with sialylated and fucosylated glycans, most preferably Thr-57, to mediate high affinity binding to P-selectin. PSGL-1 O-glycans have been determined to consist of disialylated or neutral forms of the core-2 tetrasaccharide Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcOH. A minority of the O-glycans are α1,3 fucosylated that occur as two major species containing the sialyl Lewis x antigen - one species is a disialylated, monofucosylated glycan of formula (I), and the other is a monosialylated, trifucosylated glycan having a polylactosamine backbone of formula (II), wherein R = H, OH, another sugar or an aglycone such as an amino acid.



(I)



(II)

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INTERNATIONAL SEARCH REPORT

International Application No
PCT, US 96/12820

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H3/06 C12N15/56 C12N15/79 C07K14/705 A61K31/70
A61K38/14 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 29, 22 July 1994, MD US, pages 18794-18813, XP002022672 LO-GUIDICE J.-M. ET AL: "Sialylation and Sulfation of the Carbohydrate Chains in Respiratory Mucins from a Patient with Cystic Fibrosis" see page 18802 compound IIIc1-17 ---	1
X	WO 94 11498 A (BOARD OF REGENTS OF THE UNIVERSITY OF OKLAHOMA) 26 May 1994 see page 49 - page 52 --- -/--	1-5

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
15 January 1997	10 -06- 1997
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer DAY, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT, US 96/12820

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 37, 16 September 1994, MD US, pages 23318-23327, XP002022674 MOORE K.L. ET AL: "The P-selectin Glycoprotein Ligand from Human Neutrophils Displays Sialylated, Fucosylated, O-Linked Poly-N-acetyllactosamine" see the whole document -----	1

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 96/12820

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 3-5 are directed to the treatment of the human/ animal body, the search has been carried out and based on the alleged effects of the compounds.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see annex

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1 - 5

Remark on Protest

[ ] The additional search fees were accompanied by the applicant s protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

1 . Claims 1-5 Sialylated, fucosylated O-Glycans, and their use for inhibiting inflammation mediated by P-selectin binding

2 . Claims 6-27 Proteins,protein fragments and peptides effective in inhibiting P-selectin mediated inflammation, an expression system for production of such proteins or peptides and their use for inhibiting inflammation.

INTERNATIONAL SEARCH REPORT

I. ...nation on patent family members

International Application No
PCT/... 96/12820

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9411498 A	26-05-94	AU 5727594 A	08-06-94
		CA 2151142 A	26-05-94
		EP 0668907 A	30-08-95
		US 5464778 A	07-11-95

for the lack of CTL effector function. We therefore reasoned that an Fuc-T-dependent fucosylated structure on either T cells or antigen presenting cells (APC) might be required for the generation/mediation of CTL effector function. PSGL-1 is a prominent α(1,3)-fucosylated glycoprotein expressed on APC and T cells[27]. This molecule is functionally deficient in FT -/- mice[1,2], and represents one candidate for a Fuc-T-dependent molecule required for CTL activation. Thus, we investigated the effect of soluble recombinant PSGL-1 and of PSGL-1 function blocking antibody, 2PH-1 on secondary in vitro stimulation of primed viral-specific CD8+ T cells derived from wild type mice. Wile type mice were infected with vv and on day 7 pi, their splenocytes were stimulated in vitro with vv in the absence or presence of either soluble PSGL-1 or its non-fucosylated mutant and, or PSGL-1 blocking monoclonal antibody (2PH-1)[5] or control antibodies (anti-L selectin Mel 14, or anti-human PSGL-1 antibody PL-1[28]). Both soluble PSGL-1 and function blocking anti-murine PSGL-1 antibody, but not non-fucosylated soluble PSGL-1 or control antibodies tested partially inhibited development of viral-specific CTL relative to control antibodies (Fig. 4a) 4b and data not shown). However, neither soluble PSGL-1 nor anti-murine PSGL-1 antibody had an inhibitory effect when added during the CTL assay (not shown). Thus, α(1, 3)-fucosylated PSGL or a closely related molecule appears to be required for the generation of functional CTL but not for target cell lysis.

To determine if this fucosylated molecule is required on APC or on T cells, we asked if wild type APC could activate lytic function in vv-primed FT -/-

CD8+ T cells, or if FT -/- APC were defective in their ability to activate CTL from primed wild type CD8+ T cells. Wild type and FT -/- mice were infected with vv and on day 7 pi, splenic CD8+ T cells were selected and stimulated with T cell depleted, vv-infected, γ-irradiated wild type of FT -/- splenocytes. Cytolytic function was detected in both wild type and FT -/- CD8+ cells when stimulated with wild type APC, whereas FT -/- APC were incapable of eleciting CTL activity on CD8+ cells from either wild type or FT -/- mice (Fig. 4c). Thus, a fucosylated molecule similar to PSGL-1, and expressed by APC appears to be required for effector CTL generation.

Taken together, our results suggest that APC-CD8 T cell interaction through an α(1,3)-fucosylated molecule is necessary for the development of antigen-specific CD8 CTL effector function but is not required for antigen-specific CD8 T cell proliferation or cytokine secretion. The fact that anti-murine PSGL-1 as well as soluble PSGL-1 inhibited effector function generation by wild type CD8+ T cells and that a similar defect was not seen in selectin-deficient mice suggests that PSGL-1 recognition of a counter receptor(s) that is (are) distinct from selectins is (are) required. Although PSGL-1 was originally identified as the ligand for P selectin, it is now clear that carbohydrate modifications have profound effects on its binding. Activated Ta1 cells, but not resting T cells or activated Th-2 cells, bind P-selectin, although PSGL-1 is expressed in equivalent amounts of all of these cell types[29]. Carbohydrate modifications which confer binding ability to HECA 452, an antibody directed against the cutaneous

lymphocyte antigen (CLA), modulate PSGL-1 binding to E-selectin[30]. Our results raise the possibility of additional, selectin independent receptor(s) for PSGL-1. Identification of the receptors will likely lead to insights into the mechanism of effector CTL generation and might provide tools to modify CTL killer function selectively, either to enhance it for viral infections and tumors or, to suppress it in autoimmune diseases.

Figure legend - Fig. 1

FT -/- mice are severely compromised in generating viral-specific effector CTL, but have virtually normal LAK and SEA induced CTL activity. 1a. Splenocytes from wild type or FT -/- mice infected with vv sc, and Splenocytes & PEL from mice infected ip were tested for cytolysis of vv infected MC57G (H2b) targets by 4 h Cr release assay. 1b. Splenocytes from ip infected mice were restimulated in vitro by incubation with vv infected autologous splenocytes for 5 days and tested for antiviral cytoxicity. For all the assays, background killing of uninfected MC57G targets (which was <3%) was subtracted to calculate % specific killing. 1c. Splenocytes were cultured in vitro for 3 days in the presence of either 200 IU/ml recombinant IL-S and tested for lysis of Yac-1 target cells (LAK activity) or in the presence of 10μg/ml SEA and CD8+ cells were selected and tested for lysis of Raji cells coated with SEA.

Figure legend - Fig. 2

FT -/- mice generate activated CD8+ T cells which proliferate and produce cytokines in a viral-specific manner. Splenocytes and PEL from vv infected mice

stained with FITC-conjugated and mouse Thy 1.2, CD4 or CD8 monoclonal antibodies (2a) or doubly stained with CD8 FITC or PE and CD62-L FITC, CD11α FITC or CD44 PE (2b) were analyzed by flow cytometry. For 2c and d, splenocytes from vv infected mice were immunomagnetically depleted of CD4+ T cells and NK cells and stimulated with vv as described in Fig. 1c. Three days later, culture supernatants were tested for IFN-γ levels (2c) and cells were pulsed with ^{3}H thymidine for 3 h and counted for 3 H incorporation (2d). Shown is the average +/- SEM or 3 pairs of mice.

Figure legend - Fig. 3

FT -/- but not selectin -/- mice fail to generate viral-specific effector CTL. Wile type mice and mice deficient for L-, P-, and -E selectins were infected with vv and their splenocytes were tested for antiviral cytotoxicity on day 7 pi as described in Fig. 1.

Figure legend - Fig. 4

Soluble PSGL-1 and anti-murine PSGL-1 antibody inhibits development of effector CTL by primed wild type CD8+ T cells in vitro. Splenocytes harvested from wild type mice on day 7 post vaccinia infection were stimulated with vv in the absence or presence (20 μg/ml) of soluble recombinant PSGL-1 or non fucosylated PSGL-1 (dead PSGL-1) (4a) or of anti-murine PSGL-1 antibody, 2 PH-1, or anti-human PSGL-1 antibody, PL-1 (4b). Viral-specific cytotoxicity was measured 5 days later. 4c. FT -/- APC abrogates and wild type APC restores effector CTL generation. Wild type and FT -/- mice were infected with vv and on

day 7 pi, CD8+ T cells (responders) were positively selected and stimulated with vv infected and γ-irradiated wild type or FT -/- APC (T cells depleted splenocytes). Viral-specific cytotoxicity was assayed 5 days later.

Methods

Vaccinia viral infection. FT IV and VII -/-, L-, P- and E-selectin -/- mice and their wild type counterparts were maintained under SPF facility at the Center for Blood Research. Mice 6 - 8 weeks of age and matched for sex were used for the studies. Mice were infected with WR strain of vv (ATCC) either sc at the base of the tail or ip (10^5 pfu/mice in 0.2 ml PBS).

Cytotoxicity assays. To test viral-specific cytotoxicity, on day 7 pi, peritoneal exudate cells were harvested by flushing with 3 mls of PBS and/or spleens were collected. Splenocytes and PEL were depleted of RBC by lysis in 0.17 M ammonium chloride and the cells were tested for killing of ^{51}Cr labeled, MC57G targets uninfected or infected with vv as described earlier[31]. For LAK assay, splenocytes from normal mice were cultured in the presence of 200 IU/ml recombinant IL2, and 3 days later, cells were tested for killing of ^{51}Cr labeled Yac-1 targets. For SEA induced cytotoxicity assay, splenocytes were cultured in the presence of 10μg/ml SEA (Sigma) and CD8 T cells were selected (see later) and tested for lysis of Raji cells coated with SEA (100 ng/ml for 30 min before the assay). Cytotoxicity was defined as (test release-spontaneous release)/(maximum release-spontaneous release) X 100. Percent killing of uninfected targets (vv cytotoxicity) or uncoated target (SEA induced cytoxicity) was subtracted from that

of infected/coated targets to calculate viral-specific cytotoxicity.

Antibody staining, flow cytometry and immunomagnetic depletion. To determine T cell subset numbers, splenocytes and PEL were stained singly with FTTC-conjugated anti-mouse CD3, CD4 or CD8 monoclonal antibodies (Pharmingen). Activated CD8+ T cells defined as L-selectin low, LFA-1 high and CD44 high, were assayed by dual staining with PE CD8 X FTTC Mel-14, FTTC CD11α or FTTC CD8 X PE CD44 (Pharmingen). For depletion of CD4+ T cells and NK cells, cells were stained with purified rat anti-mouse CD4 and NK 1.1 antibodies, washed and incubated with goat anti-rat Ig G coated magnetic beads (Dunai, 10 beads/cell). The depleted population contained <3% CD4 or NK cells as determined by flow cytometry.

In vitro restimulation with vv. For APC, splenocytes harvested 6-7 days post vaccinia were depleted of T cells using anti-CD3 coated Dynal beads and infected with vv (10 pfu/cell, 2 h at 37°C), irradiated (400 rads) and UV-treated as described in[32]. 5 X 10^6 infected cells were cultured with 5 X 10^6 autologous uninfected splenocytes in 24 well culture plates for 4-5 days before testing for CTL activity. In some experiments, CD4+ T cells and NK cells were depleted as described above. In some other experiments, CD8+ cells were positively selected using CD8+ milteny beads according to manufacturer's instructions. In some experiments, at the time of in vitro stimulation, soluble recombinant PSGL-1 Ig chimera, its non-fucosylated variant (dead PSGL-1) (gifts of Genetics Institute, Cambridge, MA), anti-murine PSGL-1 antibody, 2 PH-1, anti-human PSGL-1

antibody, PL-1 (gift of...), and anti-murine L-selectin antibody, Mel-14 (gift of ....) were added at a final concentration of 20 μg/ml.

Lymphocyte proliferation and IFN-γ assay. 2 X 10^5 splenocytes, depleted of CD4+ T cells and NK cells as described above, were cultured with equal numbers of γ-irradiated splenocytes that were uninfected or infected with vv in triplicate wells of 96 well trays. Three days after stimulation, 50 μg supernatants were harvested for IFN-γ assay and the cultures were pulsed with ^{3}H thymidine (0.5 μC1/well) for 6-8 h, harvested and counted for 3H incorporation as described in[27]. Supernatants were assayed for IFN-γ using IFN-γ mini assay kit (Antigen, MA, USA) calibrated with and IFN-γ standard according to manufacturers protocol.

References

1. May, P., Tail, A.D., Petryniak, B., Rogers, C.E., Smith, P.L., Marks, R.M., Kelly, R.J., Gersten, K.M., Cheng, G., Saunders, T.L., Camper, S.A., Camphausen, R.T., Sullivan, F.X., Isogai, Y., Hindsgaul, O., von Andrian & Lowe, J.B. The α(1,3) Fucosyltransferase Fuc-TVII controls leukocyte trafficking through an essential role in L-, E-, and P-selectin biosynthesis. Cell 86, 643-653 (1996).

2. Unpublished data?

3. LPE selectin KO.

4. Takada, M.K., Nadeau, K.C., Shaw, G.D., Marquette, K.A. & Tilney. The cytokine-adhesion molecule cascade in ischemia/reperfusion injury of the rat kidney. Inhibition by a soluble P-selectin ligand. J. Clin. Invest. 99, 2682-2690 (1997).

5. Borges, E., Eytner, R., Moll, T., Steegmaier, M., Matthew, A., Campbel, L.P., Ley, K., Mossmann, H. & Vestweber, D. The P-selectin glycoprotein ligand-l is important for recruitment of neutrophils into inflamed mouse peritoneum. Blood 90, 1934-1942 (1997).

6. Butcher, E.C. Leokocyte endothelial cell recognition: Three (or more) steps to specificity and diversity. Cell 67, 1033-1036 (1991).

7. Picker, L.J. Control of lymphocyte homing. Curr. Opin. Immunol. 6, 394-406 (1994).

8. Springer, T.A. Traffic signals for lymphocyte recirculation and leukocyte emigration: The multi-step paradigm. Cell 76, 301-314 (1994).

9. Hemmerich, S., Leffler, H. & Rosen, S.D. Structure of the O-Glycans in GlyCAM-1, an endothelial derived ligand for L-selectin. J. Biol. Chem. 270, 12035-12047 (1995).

10. Moore, K.L., Eaton, S.F., Lyons, D.E., Lichenstein, H.S., Cummings, R.D. & McEver, R.P. The P-selectin glycoprotein ligand from human neutrophils displays sialated, fucosylated, o-linked poly-N-acetyllactosamine. J. Biol. Chem. 269, 23318-23327 (1994).

11. Sako, D., Comess, K.M., Barone, K.M., Camphausen, R.T., Cumming, D.A., and Shaw, G.D. A sulfated peptide segment at the amino terminus of PSGL-1 is critical for P-selectin binding. Cell 83, 323-331 (1995).

12. Poutyani, T. & Seed, B. PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus. Cell 83, 333-343 (1995).

13. Li, F., Wilkins, P.P., Crawley, S., Weinstein, J., Cummings R.D. & McEver, R.P. Post-translational modifications of recombinant P-selectin glycoprotein ligand-1 required for binding to P- and E-selectin. J. Biol. Chem. 271, 3255-3264 (1996).

14. Bennink, J.R., & Yewdell, J.W. Recombinant vaccinia viruses as vectors for studying T lymphocyte specificity and function. Curr. Topics Microbiol. Immunol. 163, 153-184 (1990).

15. Spriggs, M.K., Koller, B.H., Sato, T., Crissey, P.J., Fanslow, W.C., Smithies, O., Voice, R.F., Widmer, M.B. & Maliszewski, C.R. β2-microglobulin- CD8+ T cell-deficient mice survive inoculated with high doses of vaccinia virus and exhibit alter IgG responses. Pros. Natl. Acad. Sci. USA 89, 6070-6074 (1992)

16. Binder, D., & Kundig, T.M. Antiviral Protection by CD8+ versus CD4+ T cells: CD8+ T cells correlating cytotoxicity in vitro are more efficient in anti-vacccinia protection than CD4+ dependent interleukins. J. Immunol. 146, 4301-4307 (1991).

17. Blanden, R.V. Mechanisms of recovery from a generalized viral infection: mousepox. 3. Regression of invectios foci. J. Exp Med. 133, 1091104 (1971).

18. Kagi, D., Seiler, P., Pavlovic, J. Burki, K., Zinkernagal, R.M. & Hengartner, H.

The roles of perforin- and Fas-dependent cytotoxity in protection against cytopathic and noncytopathic viruses. Eur. J. Immunol. 25, 3256-3262 (1995).

19. Muller, U., Steinhoff, U., Reis, L.F.L., Hemmi, S., Pavlovic, J., Zinkeragel, R.M., & Aguet, M. Functional rope of type I and type II interferons in antiviral defense. Science 264, 1918-1921 (1994).

20. Kagi, D., & Hengartner, H. Different roles for cytotoxic T cells in the control of infections with cytopathic versus noncytopathic viruses.

21. FT -/- T cells are severely compromised in homing to PLN - Intravital data published?

22. Tripp, R. A., Hou, S., McMickle, A., Houston, J. & Doherty, P.C. Recruitment and proliferation of CD8 T cells in respiratory virus infections. J. Immunol. 154, 6013-6021 (1995).

23. Tough, D.F., Borrow, P. & Sprent, J. Induction of bystander T cell proliferation by viruses and type 1 interferon in vivo. Science 272,1947-1950 (1996).

24. Butz, E.A. & Bevan, M.J. Massive expansion of antigen-specific CD8 T cells during an acute virus infection. Immunity 3, 167-175 (1998).

25. Counting antigen-specific CD8 T cells: A reevaluation of bystander activation during viral infection. Immunity 8, 177-187 (1998).

26. Ogg, G. S., Jin, X., Bonhoeffer, S., Dunbar, P.R., Nowak, M.A., Monard, S., Segal, J.P., Cao, Y., Rowland-Jones, S.L., Cerundolo, V., Hurley, A., Markowitz, M.., Ho, D.D., Nixon, D.F. & McMichael, A.J. Quantitation of HIV-1-specific cytotoxic t lymphocytes and plasma load of viral RNA. Science 279, 2103-2106 (1998).

27. Laszik, Z.P., Jansen, P.J., Cummings, R.D., Tedder, T.F., Mcever, R. P. & Moore, K.L. P-selectin ligand glycoprotein ligand-1 is broadly expressed in cells by myeloid, lymphoid and dentritic lineage and in some nonhematopoietic cells. Blood 88, 3010-21 (1996).

28. Norman, K.E., Moore, K.L., Mcever, R.P. & Ley, K. Leucotyte rolling in vivo is mediated by P-selectin glycoprotein-1. Blood 86, 4417-1421 (1995).

29. Borges, E., Tietz, W., Steegmaier, M.., Moil, T., Hallmann, R., Hamann, A., & Vestweber, D. P-selectin glycoprotein-1 (PSGL-1) on T helper 1 but not on T helper 2 cells bind to P-selebtin and and supports migration into inflammed skin. J. Exp. Med. 185, 573-578 (1997).

30. Fuhlbrigge, R.C., Kierfer, J.D., Armerding, D. & Kupper, T.S. Cutaneous lymphocyte antigen is a specialized form of PSGL-1 expressed on skin-homing T cells. Nature 389, 978-981 (1997).

31. Manjunath, N., Correa, M., Ardman, M. & Ardman, B. Negative regulation of T lymphocyte activation and adhesion by CD43. Nature 377, 535-538 (1995).

32. Shankar, P., Fabry, J. & Liberman, J. A simple method to selectively expand HIV-1 cytotoxic T lymphocytes in vitro. J. Immunol. Invest. 24, 489-497 (1995).

Example 2

Mice that are doubly deficient in the α(1,3)-fucosyltransferases, FT-IV and FT-VII (FT-/- mice), lack functional selectin ligands on leukocytes and endothelial cells. Here, we studied the effect of FT deficiency on CD8+ T cell responses to vaccinia virus infection. FT-/- mice developed markedly fewer cytotoxic T cells as compared to wild-type mice, although comparable numbers of CD8+ T cells accumulated at the site of infection in both strains and were capable of vigorous viral-specific proliferation. This defect in CTL generation was not due to impaired selectin-dependent T cell trafficking, because mice triply deficient in L-, P- and E-selectin developed normal antiviral cytotoxicity. Coincubation with wild-type APC induced CTL activity in primed $CD8^+$ T cells from both FT-/- and wild-type mice, whereas FT-/- APC did not induce CTL generation in either strain. CTL generation by wild-type APC was inhibited by anti-P-selectin glycoprotein ligand (PSGL)-1 and by coincubation with α(1,3)-fucosylated PSGL-1/Ig chimera, whereas non-fucosylated PSGL-1/Ig had no effect. These results suggest a novel function for PSGL-1 and perhaps other fucosylated molecules on APC in the generation of CTLs from antigen-specific $CD8^+$ T cells, which is distinct from their ability to bind selectins.

Cytotoxic T lymphocytes (CTL) are critical mediators of antigen-specific host defense against viral infections. Before a CTL response can be mounted, naive $CD8^+$ T cells must first encounter viral antigen on professional antigen-presenting cells (APCs) in secondary lymphoid organs. Antigen-activated T cells proliferate for several days and eventually migrate to the site of viral infection. Finally, they acquire effector functions, namely the ability to kill other cells that express cognate antigen on MHC class I and to produce effector cytokines, particularly interferon (IFN)-γ. The CTL response is thus dependent on the targeted movement (homing) of leukocytes in the intra- and extravascular compartments. Antigen-laden APC must initially migrate from the site of infection to organized lymphoid tissues. Here, they stimulate naïve T cells, which home to these organs from the blood. Subsequently, activated T cells must find their way back into the blood stream and from there into infected peripheral tissues.

Leukocyte migration to many lymphoid and non-lymphoid organs requires the concerted action of one or more of the three selectins (L-, E- and P-selectin, CD62) and their ligands, which are reciprocally expressed on endothelial cells and leukocytes (1-3). Selectins mediate leukocyte rolling in microvessels by binding to sialyl-$Lewis^X$ (sLeX) and related carbohydrates that are frequently presented on sialomucin scaffolds such as PSGL-1 (4,5). A critical aspect of selectin-binding carbohydrates is α(1,3)-fucosylation of one or more N-acetyl-glucosamine residues in sialylated core-2 glycans. So far, five different α(1,3)-

fucosyltransferases (FTs) have been identified in mammals, but only FT-IV and FT-VII are expressed by leukocytes and endothelial cells (6). Mice that are deficient in FT-VII have a defect in selectin-dependent leukocyte rolling and migration to sites of acute inflammation and lymphocyte homing to lymph nodes is markedly reduced (7). In contrast, FT-IV -/- mice have only a mild defect in leukocyte rolling, whereas FT-IV+VII doubly deficient (FT-/-) mice have a phenotype more severe than that of FT-VII -/- animals (8).

The importance of the selectins has been documented in many settings, including acute inflammation, atherosclerosis and cutaneous hypersensitivity responses to peripheral antigen challenge (reviewed in 2,4,5). Moreover, it has been reported that functional PSGL-1 is upregulated on many T cells after antigen recognition, and is required for their recruitment into the inflamed peritoneum (9). Correspondingly P- and E-Selectin antibodies severely compromise both CD4 and CD8 T cell recruitment to sites of acute inflammation in mice (9). However, how selectins and their ligands affect T cell recruitment and immune responses during a viral infection in vivo is not known. In particular, the role of these molecules during a CTL response to viral antigen challenge has not been examined. To address this question, we injected vaccinia virus intraperitoneally (i.p.) into FT -/- mice and animals that were triply deficient in L-, E- and P-selectin (selectin -/-) (10). Vaccinia virus has been shown to induce an acute infection in wild-type mice resulting in the generation of a robust T cell-mediated immune response and viral-specific cytotoxicity can be demonstrated directly from freshly isolated

splenocytes and peritoneal exudate lymphocytes (PEL) without restimulation in vitro (11).

All wild-type and genetically deficient animals survived the infection and virus levels became undetectable within 10 days post infection (p.i.) indicating that selectins and carbohydrates modified by FT-IV and/or FT-VII are not essential for viral clearance. However, the immune response to vaccinia virus is multi-facetted. In addition to a strong CTL response, vaccinia infection elicits natural killer (NK) cell function and IFN-γ production by NK cells, $CD4^+$ and $CD8^+$ T cells as well as a strong humoral immune response (11-17). Although $CD8^+$ CTL are the principal mediators of protection in normal animals (13), mice lacking $CD8^+$ T cells as well as mice deficient in perforin, an important component of the CTL machinery, can clear vaccinia infections (12,15,17). Therefore, normal viral clearance in mice that are deficient in FTs or selectins does not exclude that these molecules have a role in the generation, migration or function of anti-viral CTL.

Thus, we analyzed the number, composition and function of peripheral blood mononuclear cells (PBMC), PEL and splenocytes obtained from wild-type and knockout mice at day 7 p.i. Selectin -/- and FT -/- mice had much higher leukocyte counts in peripheral blood and spleen than did wild-type mice (Table 1). These results are in accordance with earlier studies that have demonstrated a role for selectins in hematopoiesis and leukocyte homeostasis (7,8,10,18). Although the frequency of $CD4^+$ T cells in blood and spleen was comparable in all strains, $CD8^+$ T cell fractions and total cell counts in these compartments were elevated in

both selectin-/- and FT-/- mice. However, at the site of infection (peritoneum), leukocyte numbers were comparable and similar numbers of $CD4^+$ and $CD8^+$ T cells were recovered in PEL from wild type and mutant mice. $CD8^+$ T cells were the most frequent subset in PEL of all strains, probably reflecting the dominance of $CD8^+$ T cell response in vaccinia infection (13). We conclude that selectin-ligand interaction is not essential for T cell migration to the inflamed peritoneal cavity in this infection model.

Table 1. also shows that equivalent fractions of T cells in the blood, spleen as well as in PEL expressed activation markers (L-selectin^{Lo} and CD44^{Hi}) suggesting that antigen-specific priming of T cells can occur normally in the absence of selectins or their ligands. During an inflammatory condition like a viral infection, not only antigen-specific T cells, but also some non-specific bystander cells may be activated and traffic to the site of infection (19,20). However, recent data using TCR transgenic mice and MHC-peptide tetramers indicate that most activated cells are indeed antigen-specific (21-23). Thus, it is likely that T cells in selectin -/- and FT -/- mice were exposed to vaccinia antigen, particularly in the spleen where selectins are not required for homing (7,24).

To determine to what extent the activated $CD8^+$ T cells in infected animals were vaccinia-specific effector cells, we tested PEL (obtained at day 7 p.i.) of infected mice for virus-specific CTL activity (25). PEL from selectin -/- mice specifically lysed virus-infected target cells at a level that was similar to wild-type controls. In contrast, PEL T cells from FT -/- mice exhibited either markedly

reduced levels of cytotoxicity (11 animals) or no detectable CTL activity (5 animals) (**Fig. 5A**). This observation suggested that FTs, but not selectins, may be required for the generation of anti-viral CTL activity in vivo. To determine whether this involved one enzyme or both, we also tested mice that were deficient in FT-IV or FT-VII alone. Both strains had significantly reduced CTL activity compared to wild-type mice, but the reduction was more notable in the FT-VII -/- than in the FT-IV -/- mice (not shown). The most striking reduction of CTL activity was seen in the FT-IV / FT-VII doubly deficient mice suggesting that both enzymes may be necessary to elicit optimal CTL activity. In additional experiments, we also tested mice that were singly deficient in P- or L-selectin (26,27) or doubly deficient in P- and E-selectin (18). Vaccinia-specific CTL activity was comparable to wild-type controls in all of these strains, which were each derived from independent ES cell clones (data not shown).

Since compromised lymphocyte trafficking seemed an unlikely explanation for the surprising diminishment of CTL in FT -/- mice, we explored two alternative hypotheses. First, FT -/- T cells might be incapable of detecting or responding to vaccinia antigen. Alternatively, antigen-specific FT -/- T cells might exist and get activated, but they may not be able to kill target cells. To test whether activated $CD8^+$ T cells in FT -/- mice recognize and respond to vaccinia-derived antigens, splenocytes were immunomagnetically depleted of $CD4^+$ T cells and NK cells, and tested for vaccinia virus-specific proliferation. $CD8^+$ T cells from primed mice proliferated rapidly and specifically upon antigen challenge

(Fig. 5B). There was no difference between $CD8^+$ T cells from FT-/- mice compared to cells from selectin -/- or WT animals. Thus, FTs are not required for the proliferative T cell response to antigen, but may be necessary later when activated $CD8^+$ T cells give rise to effector CTL.

In a separate study, we have shown that CTL activity of vaccinia-specific $CD8^+$ T cells is tightly linked to the cells' ability to produce IFN-γ in response to TCR engagement (28). Indeed, when primed FT -/- $CD8^+$ cells were treated with anti-CD3, they generated markedly reduced amounts of this effector cytokine compared to wild-type and selectin-/- $CD8^+$ cells that were stimulated in parallel (Fig. 5C). Interestingly, IFN-γ production was also reduced in FT -/- $CD4^+$ cells indicating that FT deficiency may not only affect the $CD8^+$ subset (data not shown). Thus, FT -/- $CD8^+$ cells lacked at least two distinct qualities of effector cells; CTL activity and IFN-γ production. These findings led us to hypothesize that FTs might be required to trigger one or more decisive events that must occur before activated T cells can give rise to differentiated effector cells.

The generation of Class I-restricted CTL requires interaction of $CD8^+$ T cells with APC. Thus, we asked whether FTs are required in T cells or in APC to promote CTL differentiation. We restimulated purified primed T cells from wild-type mice with APC (i.e. T cell-depleted, vaccinia virus-infected, γ-irradiated splenocytes) from FT -/- animals and *vice versa* (29). Cytolytic activity was reproducibly induced in both wild-type and FT -/- T cells that encountered vaccinia antigen presented by wild-type APC, whereas FT-/- APC were incapable

of eliciting CTL activity on $CD8^+$ cells from either wild-type or FT -/- mice (**Fig. 6**).

These results strongly suggest that one or more α(1,3)-fucosylated molecule(s) on APC induce(s) the generation of CTL from activated $CD8^+$ T cells. One of the candidate molecules we considered was PSGL-1. This sialomucin is expressed on the surface of myeloid and lymphoid cells and can be modified by FTs on many leukocytes including dendritic cells (reviewed in 5). PSGL-1 protein is expressed at normal levels on FT -/- leukocytes, but it is functionally deficient because it lacks the fucosylation needed to serve as a selectin ligand (8 and data not shown) . To assess whether fucosylated PSGL-1 was involved in CTL differentiation, we took two approaches. First, we harvested primed splenocytes from vaccinia infected wild-type mice on day 7 p.i. and restimulated the cells with wild-type APC for five days in the presence of mAb 2PH-1 to the N-terminus (aa 42-60) of murine PSGL-1 (30,31). This mAb significantly inhibited CTL generation, whereas mAb Mel-14 to murine L-selectin (32) had no effect (**Fig. 7A**). Second, we exposed primed $CD8^+$ T cells to vaccinia virus-infected wild-type APC in the presence of a soluble protein consisting of the 40 N-terminal amino acids of human PSGL-1 linked to human Ig heavy chain (PSGL-1/Ig) (33). Recombinant PSGL-1/Ig was either generated in cells that had been cotransfected with core-2 enzyme and FT-VII (to generate PSGL-1/Ig decorated with sLeX-like carbohydrates or from cells that expressed only core-2 enzyme, but not FT-VII (mimicking non-fucosylated PSGL-1 in FT -/- mice). Coincubation with the

fucosylated PSGL-1/Ig partially blocked the generation of viral-specific CTL, whereas non-fucosylated PSGL-1/Ig had no effect (**Fig. 7B**). Importantly, inhibitors of PSGL-1 were only effective when they were present during T cell stimulation by APC. Neither anti-PSGL-1 nor fucosylated PSGL-1/Ig inhibited target cell lysis when they were added only during the CTL assay (not shown).

These findings demonstrate a novel physiological role for the α(1,3)-fucosyltransferases, FT-IV and FT-VII, in APC. Our data suggest that FTs exert this pivotal role by decorating surface-expressed glycoproteins on APC, one of which is PSGL-1. Since anti-PSGL-1 and PSGL-1/Ig were only partially effective in blocking the in vitro generation of CTL from primed wild-type $CD8^+$ cells, it cannot be excluded that additional fucosylated molecules exist on APC that may play a similar role. However, mAb 2PH-1 was originally raised against a synthetic peptide resembling the N-terminus of murine PSGL-1 and was selected to block P-selectin/PSGL-1 interactions (30). The finding that CTL activity was normal in selectin -/- mice suggests that activated $CD8^+$ cells express counter-receptor(s) for PSGL-1 that must be distinct from the known selectins. It is therefore possible that the hypothetical receptor(s) engage(s) PSGL-1 in a manner that is not entirely inhibitable by mAb 2PH-1. In any event, our results indicate that the manipulation of FTs or PSGL-1 on APC or the putative PSGL-1 receptor(s) on T cells will be useful to control the generation of $CD8^+$ effector T cells. This may prove to be a powerful tool to learn more about the generation and function of CTL in vivo. Moreover, our findings may offer a new approach to treat pathologic conditions in

humans that are associated with abnormal generation or function of CTL. For example, the ability to selectively modify this critical step might be useful to enhance CTL killer function during viral infections or to combat tumors, whereas CTL suppression might be beneficial for the treatment of autoimmune diseases.

References

1. E.C. Butcher, L.J. Picker. Science **272**, 60
2. T.M. Carlos, J.M. Harlan. Blood **84**, 2068 (1994).
3. T.A. Springer. Cell **76**, 301 (1994).
4. D. Vestweber, J.E. Blanks. Physiol. Rev. **79**, 181 (1999).
5. G.S. Kansas. Blood **88**, 3259 (1996)
6. J.B. Lowe. Kidney Int. 51, 1418 (1997)
7. P. Maly, A.D. Thall, B. Petryniak, C.E. Rogers, P.L. Smith, R.M. Marks, R.J. Kelly, K.M. Gersten, G. Cheng, T.L. Saunders, S.A. Camper, R.T. Camphausen, F.x. Sullivan, Y. Isogai, O. Hindsgaul, U.H. von Andrian, J.B. Lowe. Cell **86**, 643 (1996).
8. John Lowe Unpublised data
9. H. Xie, Y.C. Lim, Luscinskas, A.H. Lichtman. J. Exp. Med. **189**, 1765 (1999)
10. S.D. Robinson, P.S. Frenette, H. Rayburn, M. Cummiskey, M. Ullman-Cullere, D.D. wagner, R.O. Hynes. Oroc. Natl. Acad. Sci. USA in press
11. J. R. Bennink, J.W. Yewdell. Curr. Topics Microbiol. Immunol. **163**, 153 (1990).
12. M.K. Spriggs, B.H. Koller, T. Sato, P.J. Orrissey, W.C. Fanslow, O. Smithies, R.F. Voice, M.B. Widmer, C.R. Maliszewski. Proc. Natl. Acad. Sci. USA. **89**, 6070 (1992).
13. D. Binder, T.M. Kundig. J. Immunol. **146**, 4301 (1991).
14. R.V. Blanden. J. Exp. Med. **133**, 1091, (1971)
15. D. Kagi, P. Seiler, J. Pavlovic, K. Burki, R.M. Zinkernagel, H. Hengartner.

Eur. J. Immunol. **25**, 3256 (1995).

16. U. Muller, U. Steinhoff, L.F.L Reis, S. Hemmi, J. Pavlovic, R.M. Zinkernagel, M. Aguet. Science **264,** 1918 (1994)

17. D. Kagi, H. Hengartner Curr. Opin. Immunol. **8**, 472 (1996)

18. P.S. Frenette, T.N. Mayadas, H. Rayburn, R.O. Hynes, D.D. Wagner. Cell **84**, 563 (1996)

19. R.A. Tripp, S. Hou, A. McMickle, J. Houston, P.C. Doherty. J. Immunol. **154**, 6013 (1995).

20. D.F. Tough, P. Borrow, J. Sprent. Science **272**, 1947 (1996).

21. E.A. Butz, M.J. Bevan. Immunity **8**, 167 (1998).

22. K. Murali-Krishna, J.D. Altman, M. Suresh, D.J.D. Sourdive, A.J. Zajec, J.D. Miller, J. Slansky, R. Ahmed. Immunity **8**, 177 (1998).

23. G.S. Ogg, X. Jin, S. Bonhoeffer, P.R. Dunbar, M.A. Nowak, S. Monard, J.P. Segal, Y. Cao, S.L. Rowland-Jones, V. Cerundolo, A. Hurley, M. Markowitz, D.D. Ho, D.F. Nixon, A.j. McMichael. Science **279**, 2103 (1998).

24. M.L. Arbones, D.C. Old, K. Ley, H. Ratech, C. Maynard-Curry, G. Otten, D.J. Capon, T.F. Tedder. Immunity **1**, 247 (1994)

25. Wild-type, FT -/-, and selectin -/- mice (6-8 weeks of age and matched for sex) were infected with the WR strain of vv (ATCC) either sc at the base of the tail or ip (10^5 pfu/mice in 0.2 ml PBS). On day 7 pi, PEL were harvested by flushing with 3 mls of PBS and /or spleens were collected. Splenocytes and PEL were depleted of RBC by lysis in 0.17 M ammonium chloride and tested

for killing of ^{51}Cr labeled, MC57G targets, uninfected or infected with vv in a standard chromium release assay. Cytotoxicity was defined as (test release-spontaneous release)/ (maximum release-spontaneous release) X 100%. Percent killing of uninfected targets was subtracted from that of infected targets to calculate viral-specific cytotoxicity.

26. T.N. Mayadas, R.C. Johnson, H. Rayburn, R.O. Hynes, D.D. Wagner. Cell **74**, 541, (1993).

27. M.D. Catalina, M.C. Carroll, H. A. Arizpe, A. Takashima, P. Estess, M.H. Siegleman. J. Exp. Med. **184**, 2341 (1996).

28. N. Manjunath, P. Shankar, J. Lieberman, U.H. von Andrian. Submitted

29. Mice were infected with vv ip and seven days later, $CD8^+$ T cells were positively selected using anti-CD8 antibody-coated Miltenyi beads according to manufacturer's instructions. For APC, splenocytes were depleted of T cells using anti-CD3 coated Miltenyi beads, infected with vv (10 pfu/cell, 2 h at 37^0C), irradiated (400 rads) and UV-treated as described earlier (34). $2X10^6$ $CD8^+$ T cells obtained from wild-type or FT-/- mice were cultured with $5X10^5$ wild-type and FT-/- APC in 24-well culture plates for 4-5 days before testing for CTL activity.

30. E. Borges, R. Eytner, R, T. Moll, M. Steegmaier, A. Matthew, I.P. Campbel, K. Ley, H. Mossmann, D. Vestweber. Blood **90**, 1934 (1997).

31. Wild type mice were infected with vv ip and 7 days later, splenic $CD8^+$ T cells were restimulated with vv- infected APC in 24-well plates as described in ref.

29. At the time of in vitro stimulation, in some cultures soluble recombinant PSGL-1 Ig chimera, its non-fucosylated variant, anti-murine PSGL-1 antibody, 2PH-1, or anti-murine L-selectin antibody, Mel-14 were added at a final concentration of 20 μg/ml. Viral-specific cytotoxicity was determined after 5 days of culture.

32. W.M. Gallatin, I.L. Weissman, E.C Butcher. Nature **304**, 30 (1983).

33. M.K. Takada, K.C. Nadeau, G.D. Shaw, K.A. Marquette, Tilney. J. Clin. Invest. **99**, 2682 (1997).

34. P. Shankar, J. Fabry, J. Lieberman. Immunol. Invest. **24**, 489 (1995).

TABLE AND FIGURE LEGENDS

Table 1

Total leukocyte counts, T cell subset frequency and activation status of $CD8^+$ T cells in PEL, spleen and peripheral blood of wild-type, selectin -/- and FT -/- mice. Mice were infected by i.p. injection of vaccinia virus (10^5 pfu/mouse) and at day 7 p.i., peripheral blood was obtained by tail bleeding and PEL and spleen were harvested. After lysing of RBC, leukocyte counts were performed on all samples using a hemocytometer. To determine T cell subset proportions, aliquots of cells were labeled with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 and analyzed on a flow cytometer (FACScan, Becton Dickinson) following standard procedures. To determine the activation status, cells were labeled with anti-CD8 FITC and anti-L-selectin PE or anti-CD8 FITC and anti-CD44 PE. Shown are % $CD8^+$ T cells that were L-selectin low or CD44 high. L-

selectin levels are not shown for selectin-/- mice because all cells were negative for L-selectin. Mean+/- SD from 6 mice in each group are shown.

Fig. 5

Anti-viral CTL activity and IFN-γ production but not virus-specific proliferation is markedly reduced in FT-/- mice. **5A.** CTL activity is reduced in FT-/- but not in selectin-/- mice. Wild-type, triple selectin-/- and FT-/- mice were infected with vv ip and 7 days later, their PEL were tested for lysis of vv infected ^{51} Cr labeled MC57G target cells (25). Scattergrams for 16 wild-type, 16 FT-/- and 10 selectin-/- mice at 4 different effector: target (E:T) ratios are shown. Each symbol represents the mean percent specific cytotoxicity (from triplicate measurements) of cells from a single animal. **5B.** Viral-specific proliferation is comparable in selectin-/- and FT-/- mice. Mice were infected with vv and 7 days later, their splenocytes were immunomagnetically depleted of $CD4^+$ T cells and NK cells. $2x10^5$ depleted splenocytes were cultured with equal numbers of T cell-depleted and γ-irradiated splenocytes that were uninfected or infected with vv in triplicate wells of 96-well plates. Two days after stimulation, the cultures were pulsed with ^{3}H thymidine (0.5 μCi/well) for 6-8 h, harvested and counted for ^{3}H incorporation. Shown is the mean cpm +/- S.D. from 3 mice for each strain. **5C.** IFN-γ_production is reduced in FT-/- mice but not in selectin-/- mice. PEL obtained on d7 pi were stimulated with 1 μg/ml αCD3 in the presence of Brefeldin A for 6 h, stained with anti-CD8 Cychrome, fixed, permeabilized and then stained with anti-IFN-γ_PE using intracellular staining kit

(Pharmingen) before analyzing in a flow cytometer. Representative results from 1 mouse for each strain (out of 3 animals tested with similar results) are shown.

Fig. 6

α(1,3)-fucosylated PSGL-1 is required on APC for the induction of CTL activity in activated $CD8^+$ cells.

Wild-type and FT-/- mice were infected with vv and 7 days later, their splenic $CD8^+$ T cells were immunomagnetically selected and stimulated with vv-infected wild-type or FT-/- APC (T cell depleted, γ-irradiated splenocytes). Cytotoxicity was measured after 5 days of culture as described in Fig. 5 and ref.25. Results from 2 mice for each strain are shown.

Fig. 7

Secondary stimulation of CTL activity in primed wild-type $CD8^+$ T cells is specifically attenuated in the presence of PSGL-1 blocking antibody or in the presence of recombinant α(1,3)-fucosylated PSGL-1. Wild-type mice were infected with vv and 7 days later, splenocytes were harvested and stimulated with vv in the absence or presence of 20μg/ml blocking anti-PSGL-1 antibody, 2 PH-1, or control anti-L-selectin antibody, Mel-14 (**7A**) or in the presence of soluble recombinant fucosylated or non-fucosylated PSGL-1-Ig (**7B**). Cytotoxicity was determined after 5 days of culture as described in Fig.5 and ref.25. Results from four individual mice for **7A** and three mice for **7B** are shown.

Acknowledgments

This work was supported by National Institute of Health grants HL54936, HL 02881 and HL41484.

Table 1

Total Cells	Blood $x10^6$/ml ± S.D.	PEL $x10^6$ ± S.D.	Spleen $x10^5$ ± S.D.
+/+	5.8 ± 0.8	16.1 ± 3.1	117.5 ± 11.0
Selectin -/-	17.5 ± 2.0	19.2 ± 3.3	262.5 ± 59.0
FT -/-	20.7 ± 6.3	20.75 ± 5.5	300 ± 88
$CD4^+$ T cells	Percent Total ± S.D.		
+/+	12.3 ± 5.5	22.9 ± 5.0	17.2 ± 2.1
Selectin -/-	9.24 ± 2.7	18.3 ± 4.1	17.5 ± 3.5
FT -/-	10.1 ± 3.2	23.8 ± 4.6	21.4 ± 4.7
$CD8^+$ T cells	Percent Total ± S.D.		
+/+	11.1 ± 7.2	42.4 ± 14.0	10.6 ± 2.6
Selectin -/-	19.8 ± 7.3	50.6 ± 11.5	20.9 ± 8.0
FT -/-	19.3 ± 8.0	56.5 ± 6.1	21.3 ± 4.6
Activation Status of CD8 T cells			
	L-selectin low / CD44 high	L-selectin low / CD44 high	L-selectin low / CD44 high
+/+	73 ± 8 / 86 ± 5	85 ± 7 / 96 ± 1	58 ± 5 / 59 ± 3
Selectin -/-	89 ± 6	98 ± 2	62 ± 3
FT -/-	79 ± 5 / 92 ± 3	83 ± 3 / 98 ± 1	54 ± 5 / 61 ± 7

Claims:

1. A method of inhibiting the differentiation of an activated T-cell into a cytotoxic lymphocyte in a mammalian subject, said method comprising administering to said subject a therapeutically effective amount of a PSGL antagonist.

2. The method of claim 1, wherein said PSGL antagonist is selected from the group consisting of a soluble form of PSGL, an antibody directed to PSGL, an antibody directed to sLe_x, an antibody directed to sulfated tyrosine, sLe_x, mimetics which inhibit sLe_x binding and a small molecule inhibitor of PSGL binding.

3. The method of claim 2, wherein said PSGL antagonist is a soluble form of PSGL.

4. The method of claim 2, wherein said PSGL antagonist is an antibody directed to PSGL.

5. A method of treating or ameliorating an autoimmune condition, said method comprising administering to said subject a therapeutically effective amount of a PSGL antagonist.

6. The method of claim 5, wherein said PSGL antagonist is selected from the group consisting of a soluble form of PSGL, an antibody directed to PSGL, an antibody directed to sLe_x, an antibody directed to sulfated tyrosine, sLe_x, mimetics which inhibit sLe_x binding and a small molecule inhibitor of PSGL binding.

7. The method of claim 6, wherein said PSGL antagonist is a soluble form of PSGL.

8. The method of claim 6, wherein said PSGL antagonist is an antibody

directed to PSGL.

9. A method of treating or ameliorating a allergic reaction, said method comprising administering to said subject a therapeutically effective amount of a PSGL antagonist.

10. The method of claim 9, wherein said PSGL antagonist is selected from the group consisting of a soluble form of PSGL, an antibody directed to PSGL, an antibody directed to sLe_x, an antibody directed to sulfated tyrosine, sLe_x, mimetics which inhibit sLe_x binding and a small molecule inhibitor of PSGL binding.

11. The method of claim 10, wherein said PSGL antagonist is a soluble form of PSGL.

12. The method of claim 10, wherein said PSGL antagonist is an antibody directed to PSGL.

13. A method of treating or ameliorating asthma, said method comprising administering to said subject a therapeutically effective amount of a PSGL antagonist.

14. The method of claim 13, wherein said PSGL antagonist is selected from the group consisting of a soluble form of PSGL, an antibody directed to PSGL, an antibody directed to sLe_x, an antibody directed to sulfated tyrosine, sLe_x, mimetics which inhibit sLe_x binding and a small molecule inhibitor of PSGL binding.

15. The method of claim 14, wherein said PSGL antagonist is a soluble form of PSGL.

16. The method of claim 14, wherein said PSGL antagonist is an antibody directed to PSGL.

17. The method of claim 3, wherein said soluble form of PSGL comprises the first 19 amino acids of the mature amino acid sequence of PSGL.

18. The method of claim 17, wherein said soluble form of PSGL comprises the first 47 amino acids of the mature amino acid sequence of PSGL.

19. The method of claim 18, wherein said 47 amino acids are fused to the Ig portion of an immunoglobulin chain.

20. The method of claim 7, wherein said soluble form of PSGL comprises the first 19 amino acids of the mature amino acid sequence of PSGL.

21. The method of claim 20, wherein said soluble form of PSGL comprises the first 47 amino acids of the mature amino acid sequence of PSGL.

22. The method of claim 21, wherein said 47 amino acids are fused to the Ig portion of an immunoglobulin chain.

23. The method of claim 11, wherein said soluble form of PSGL comprises the first 19 amino acids of the mature amino acid sequence of PSGL.

24. The method of claim 23, wherein said soluble form of PSGL comprises the first 47 amino acids of the mature amino acid sequence of PSGL.

25. The method of claim 24, wherein said 47 amino acids are fused to the Ig portion of an immunoglobulin chain.

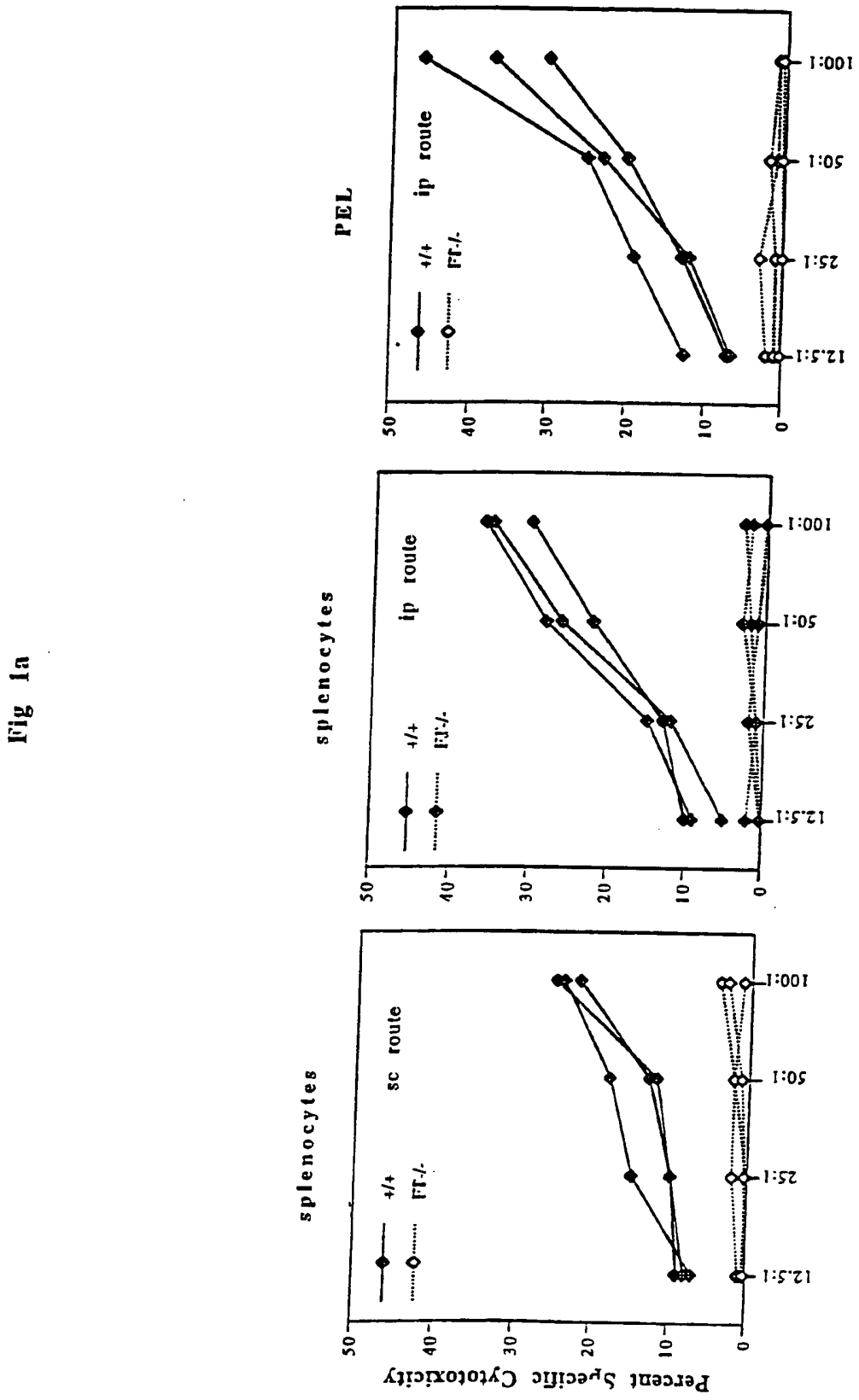

Percent Specific Cytotoxicity
splenocytes
sc route
+/+
FT-/-
splenocytes
ip route
+/+
FT-/-
PEL
ip route
+/+
FT-/-
0
10
20
30
40
50
12.5:1
25:1
50:1
100:1
E:T Ratio

Fig 1a

Fig 1b

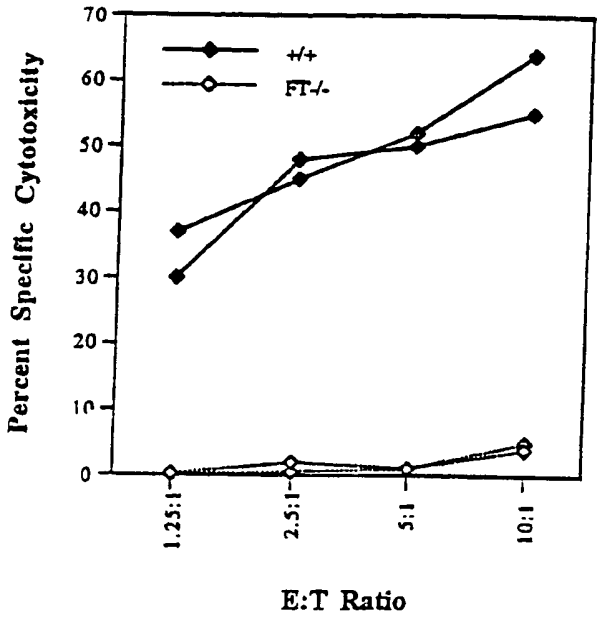

70
60
50
40
30
20
10
0
+/+
FT-/-
Percent Specific Cytotoxicity
1.25:1
2.5:1
5:1
10:1
E:T Ratio

Fig. 1c.

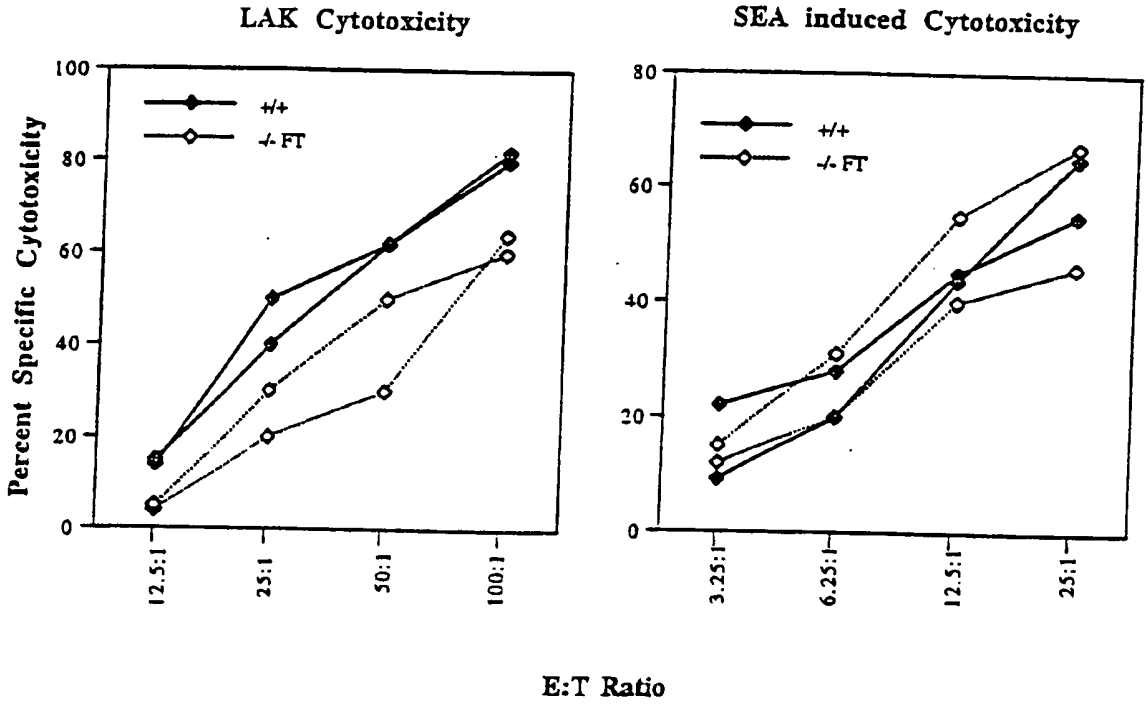
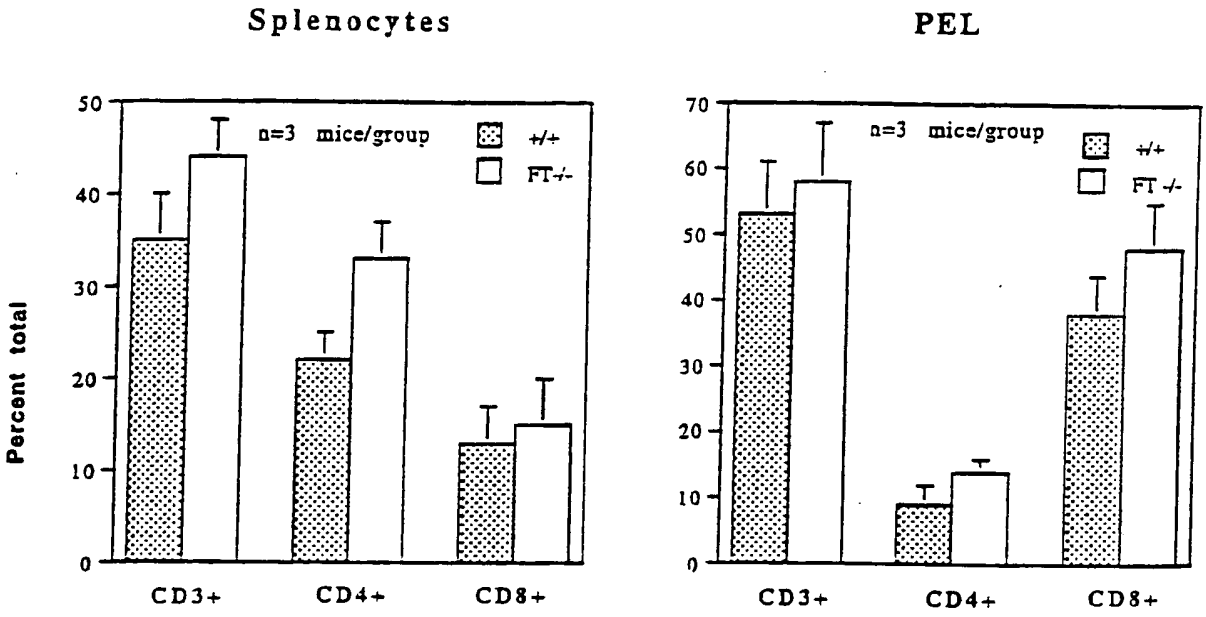

LAK Cytotoxicity
SEA induced Cytotoxicity
Percent Specific Cytotoxicity
+/+
-/- FT
100
80
60
40
20
0
12.5:1
25:1
50:1
100:1
3.25:1
6.25:1
12.5:1
25:1
E:T Ratio

Fig 2a


Splenocytes
PEL
Percent total
n=3 mice/group
+/+
FT-/-
CD3+
CD4+
CD8+
0
10
20
30
40
50
60
70

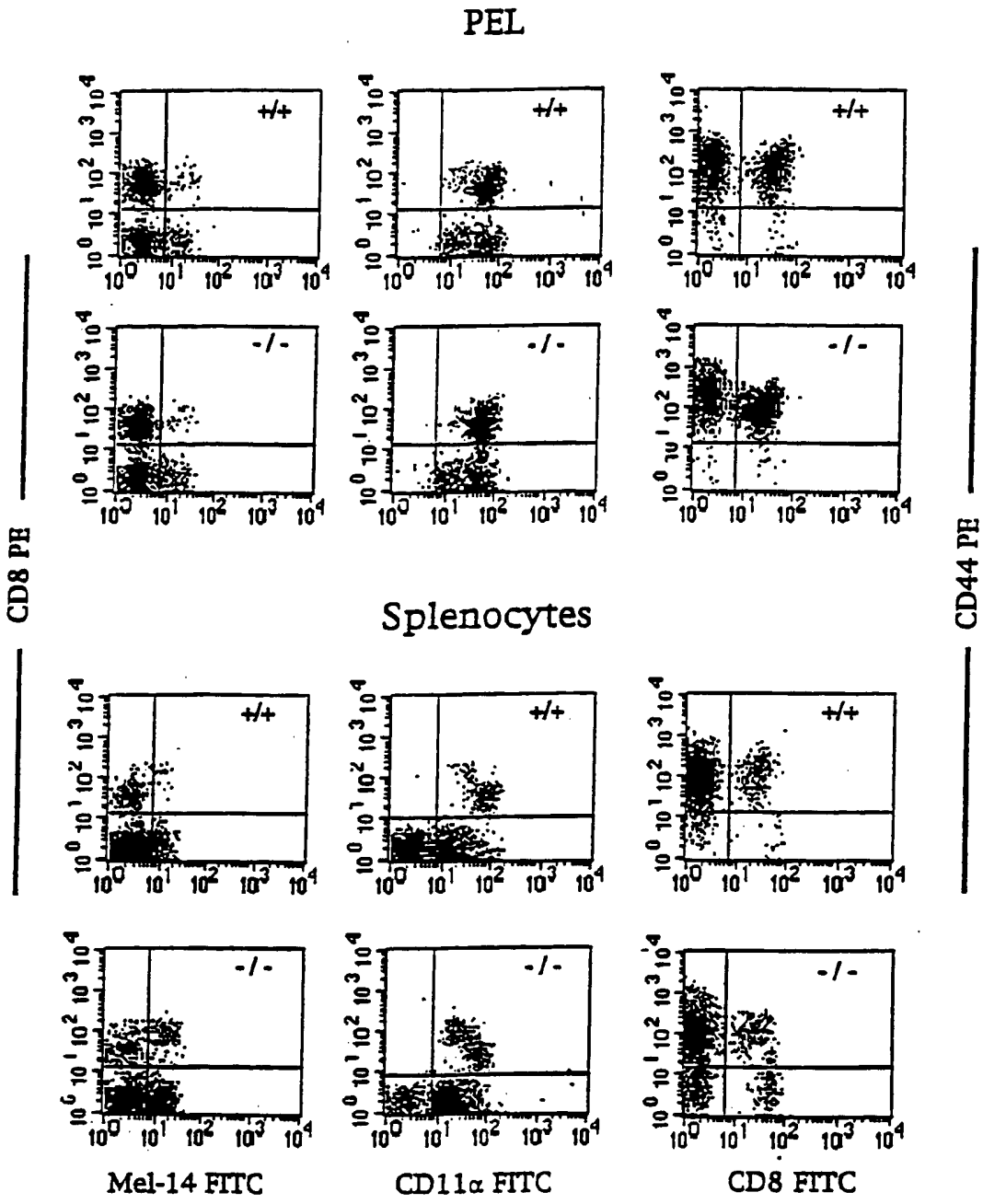

PEL
+/+
-/-
CD8 PE
CD44 PE
Splenocytes
+/+
-/-
Mel-14 FITC
CD11α FITC
CD8 FITC

Fig 2B

Fig 2c

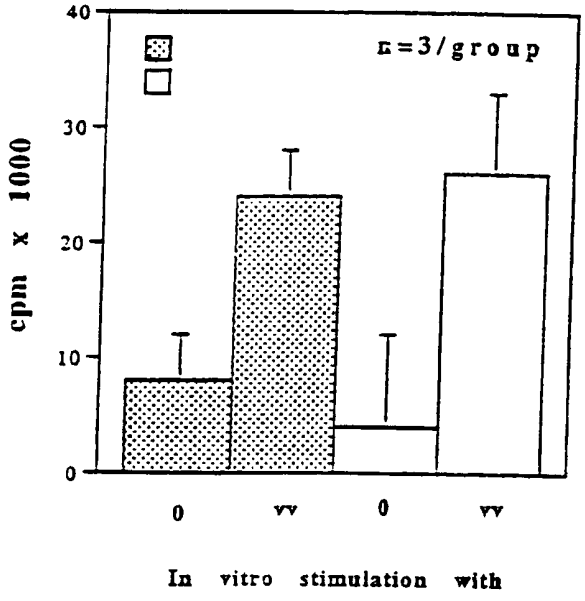

40
30
20
10
0
cpm x 1000
n=3/group
0
vv
0
vv
In vitro stimulation with

Fig 2d

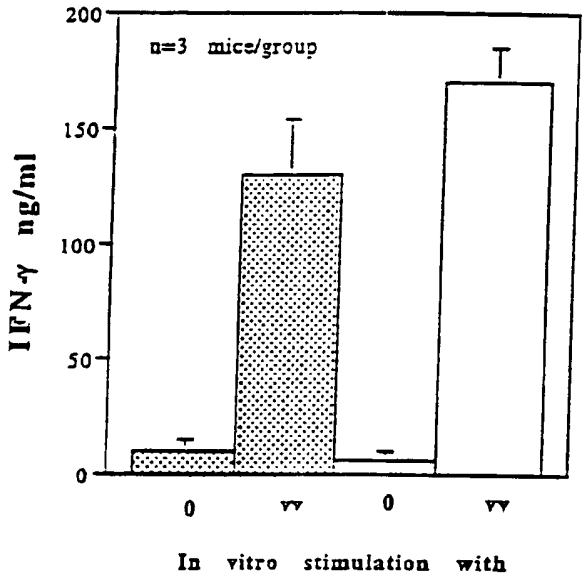

200
150
100
50
0
n=3 mice/group
IFN-γ ng/ml
0
vv
0
vv
In vitro stimulation with

Fig 3

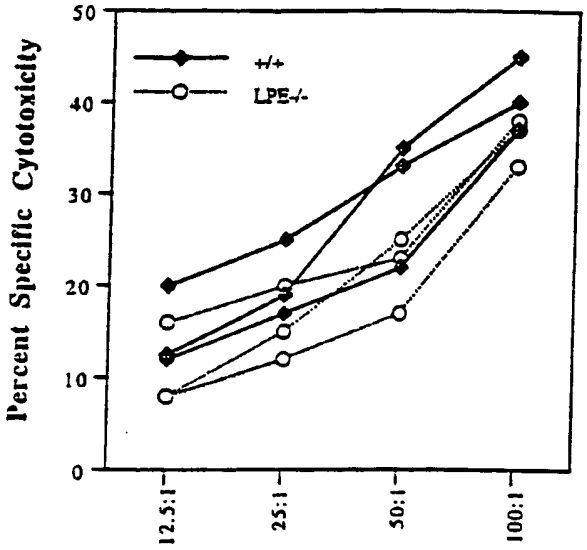

Percent Specific Cytotoxicity
50
40
30
20
10
0
+/+
LPE-/-
12.5:1
25:1
50:1
100:1
E:T Ratio

Fig 4a

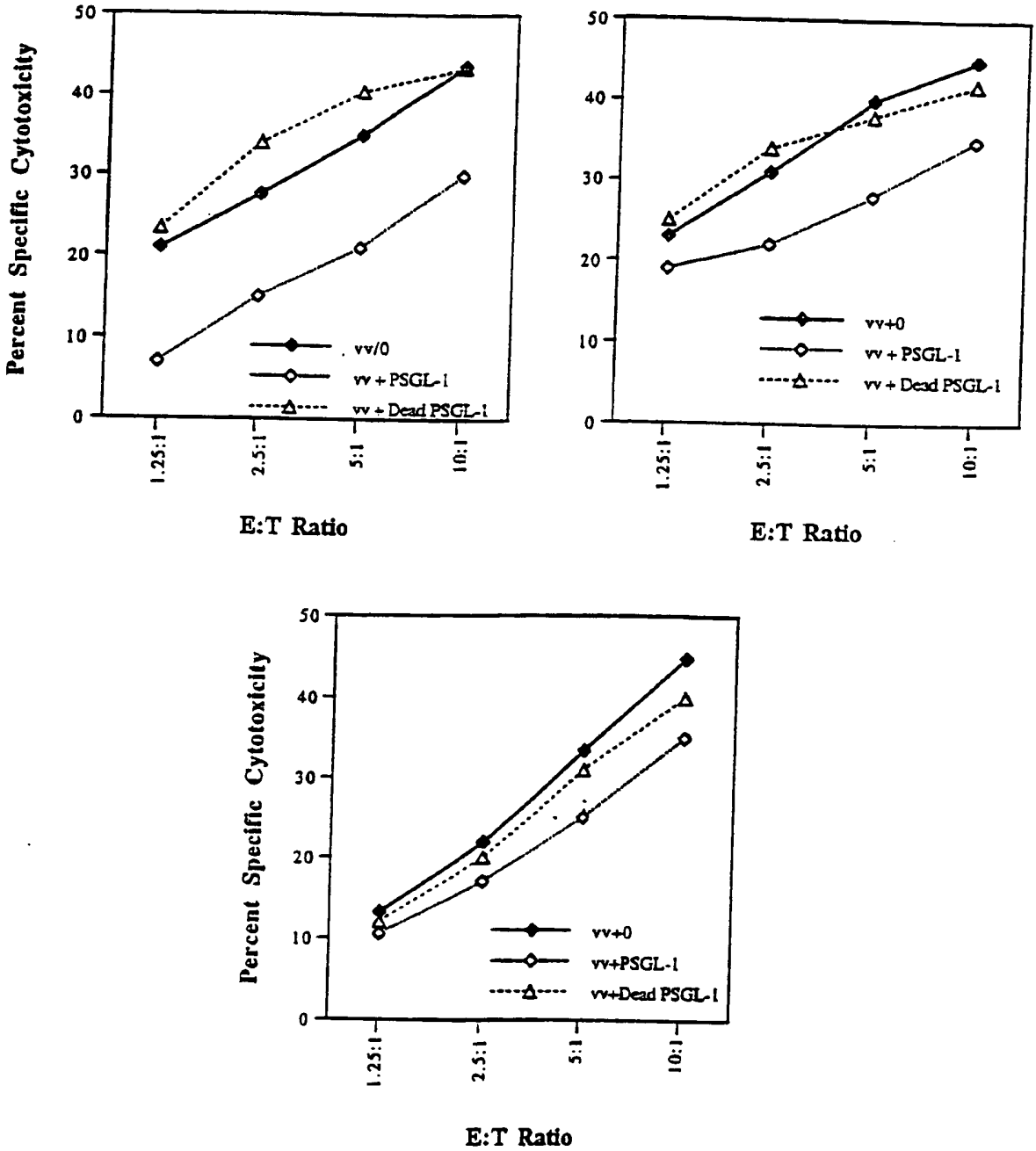

Percent Specific Cytotoxicity
50
40
30
20
10
0
vv/0
vv + PSGL-1
vv + Dead PSGL-1
1.25:1
2.5:1
5:1
10:1
E:T Ratio
vv+0
vv + PSGL-1
vv + Dead PSGL-1
1.25:1
2.5:1
5:1
10:1
E:T Ratio
Percent Specific Cytotoxicity
50
40
30
20
10
0
vv+0
vv+PSGL-1
vv+Dead PSGL-1
1.25:1
2.5:1
5:1
10:1
E:T Ratio

Fig. 4b

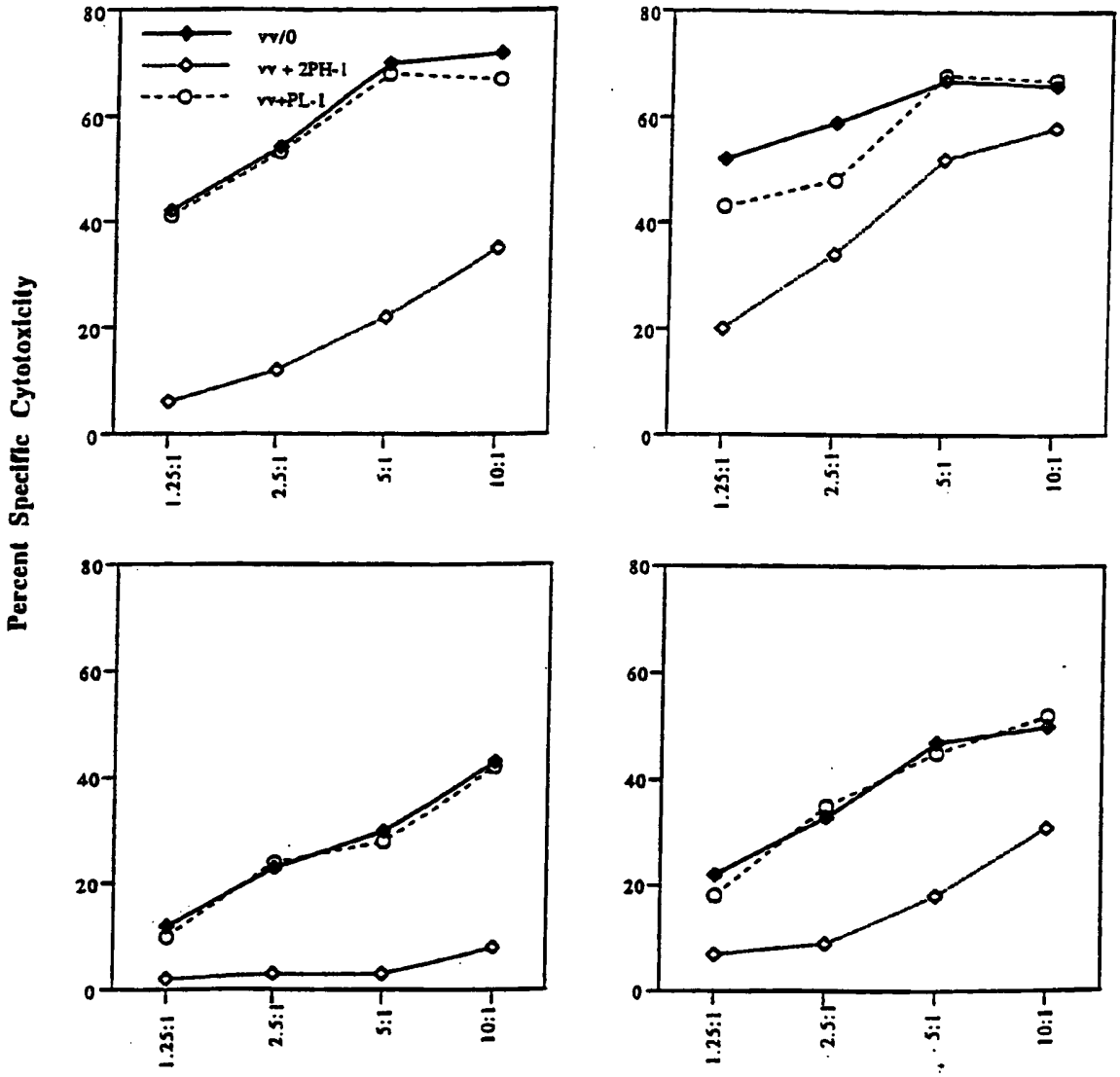

vv/0
vv + 2PH-1
vv+PL-1
Percent Specific Cytotoxicity
80
60
40
20
0
1.25:1
2.5:1
5:1
10:1
E:T Ratio

Fig. 4 c

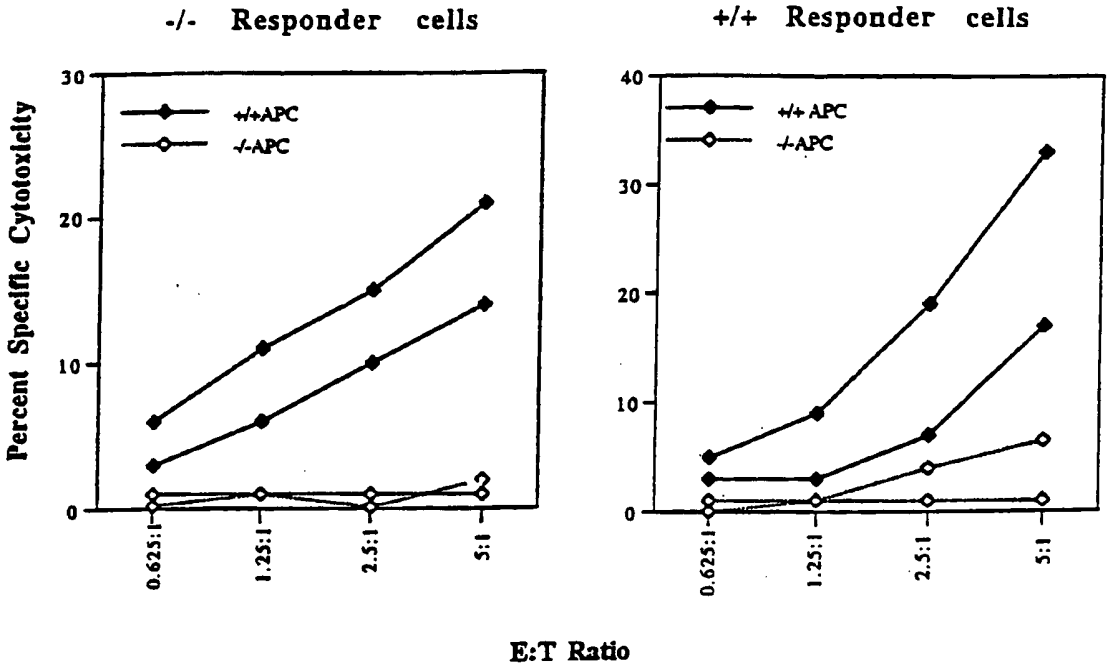

-/- Responder cells
+/+ Responder cells
Percent Specific Cytotoxicity
+/+APC
-/-APC
+/+ APC
-/-APC
0
10
20
30
40
0.625:1
1.25:1
2.5:1
5:1
E:T Ratio

Fig. 5A

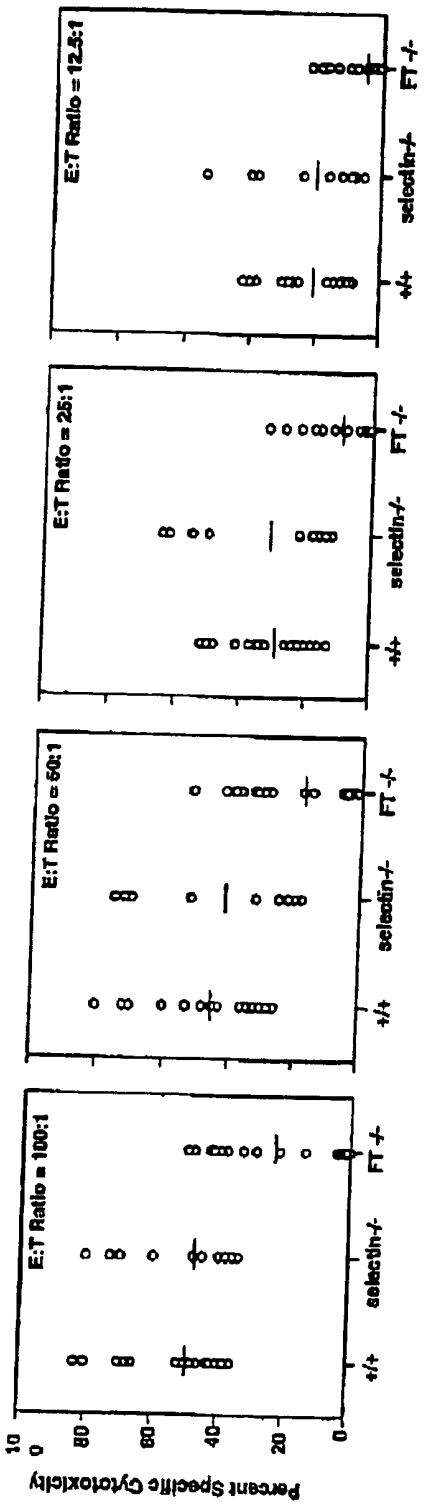

Percent Specific Cytotoxicity
0
20
40
60
80
100
E:T Ratio = 100:1
E:T Ratio = 50:1
E:T Ratio = 25:1
E:T Ratio = 12.5:1
+/+
selectin-/-
FT -/-

Fig. 5B

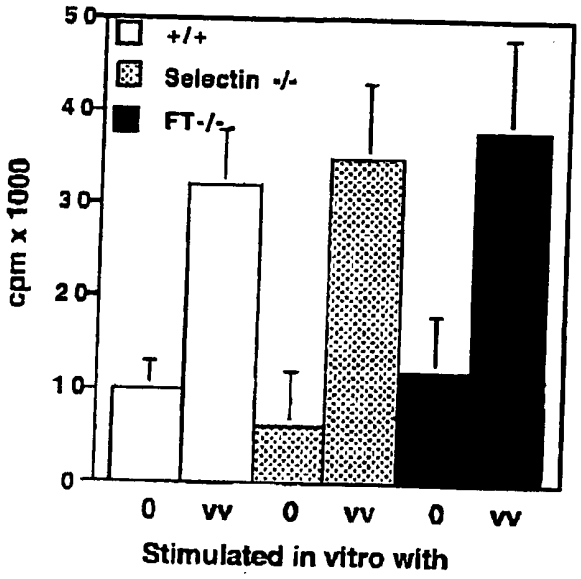

50
40
30
20
10
0
cpm x 1000
+/+
Selectin -/-
FT-/-
0
vv
0
vv
0
vv
Stimulated in vitro with

Fig 5C

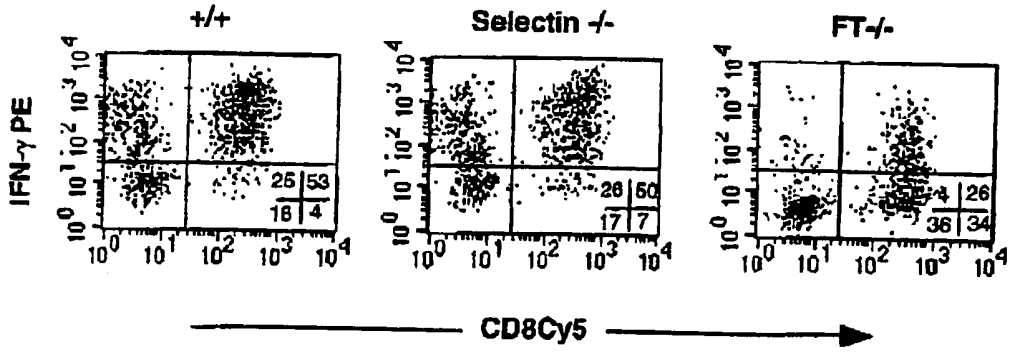


Fig. 6

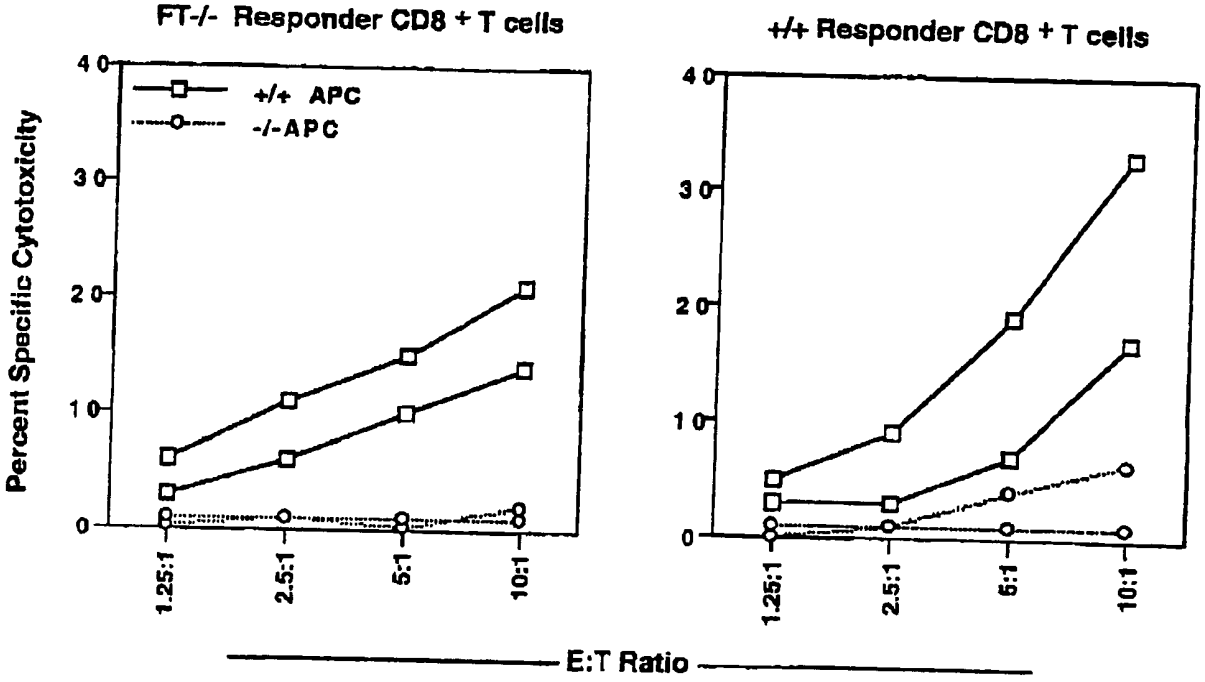

FT-/- Responder CD8 + T cells
+/+ Responder CD8 + T cells
+/+ APC
-/-APC
Percent Specific Cytotoxicity
40
30
20
10
0
1.25:1
2.5:1
5:1
10:1
E:T Ratio

Fig. 7A

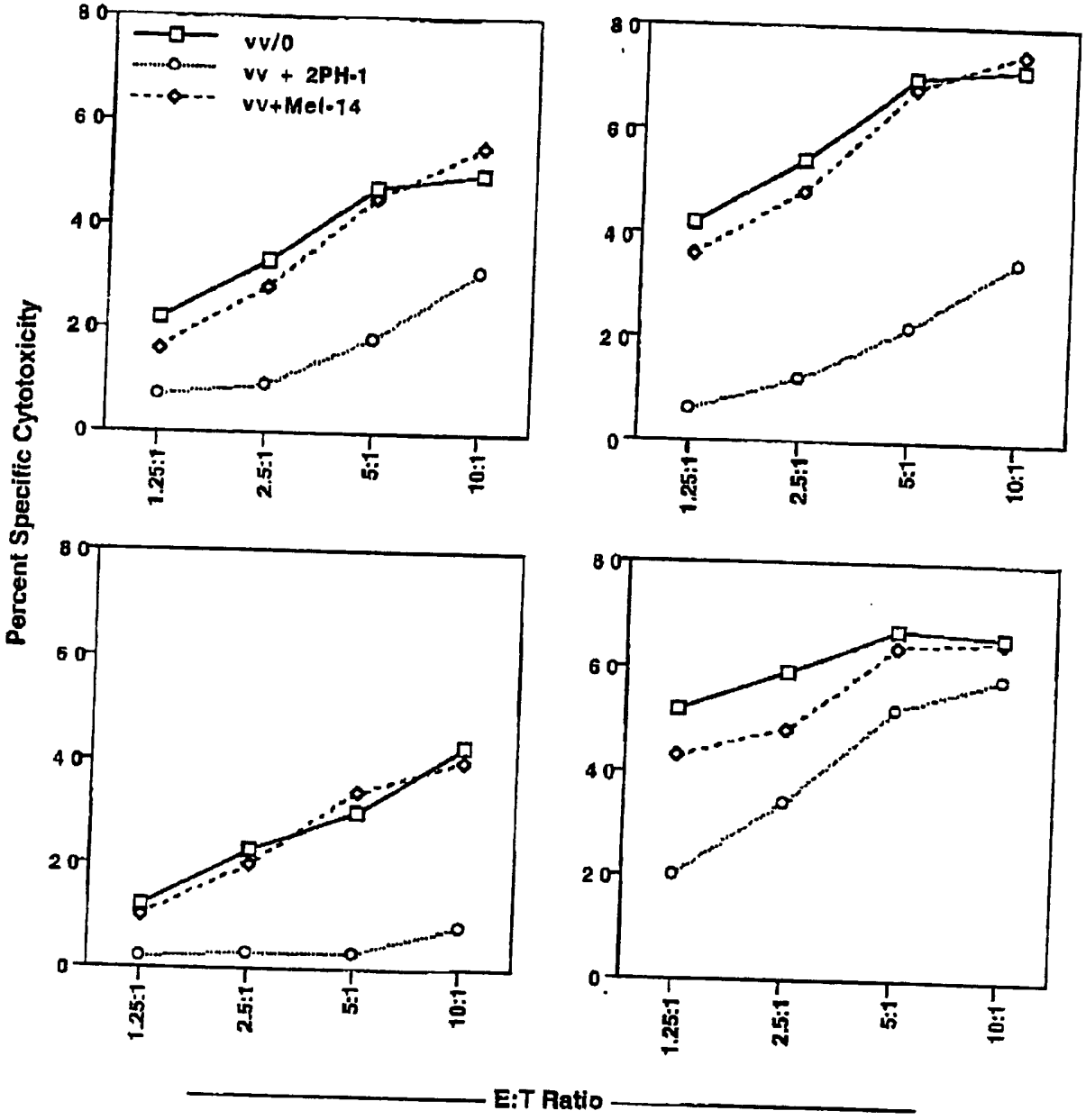
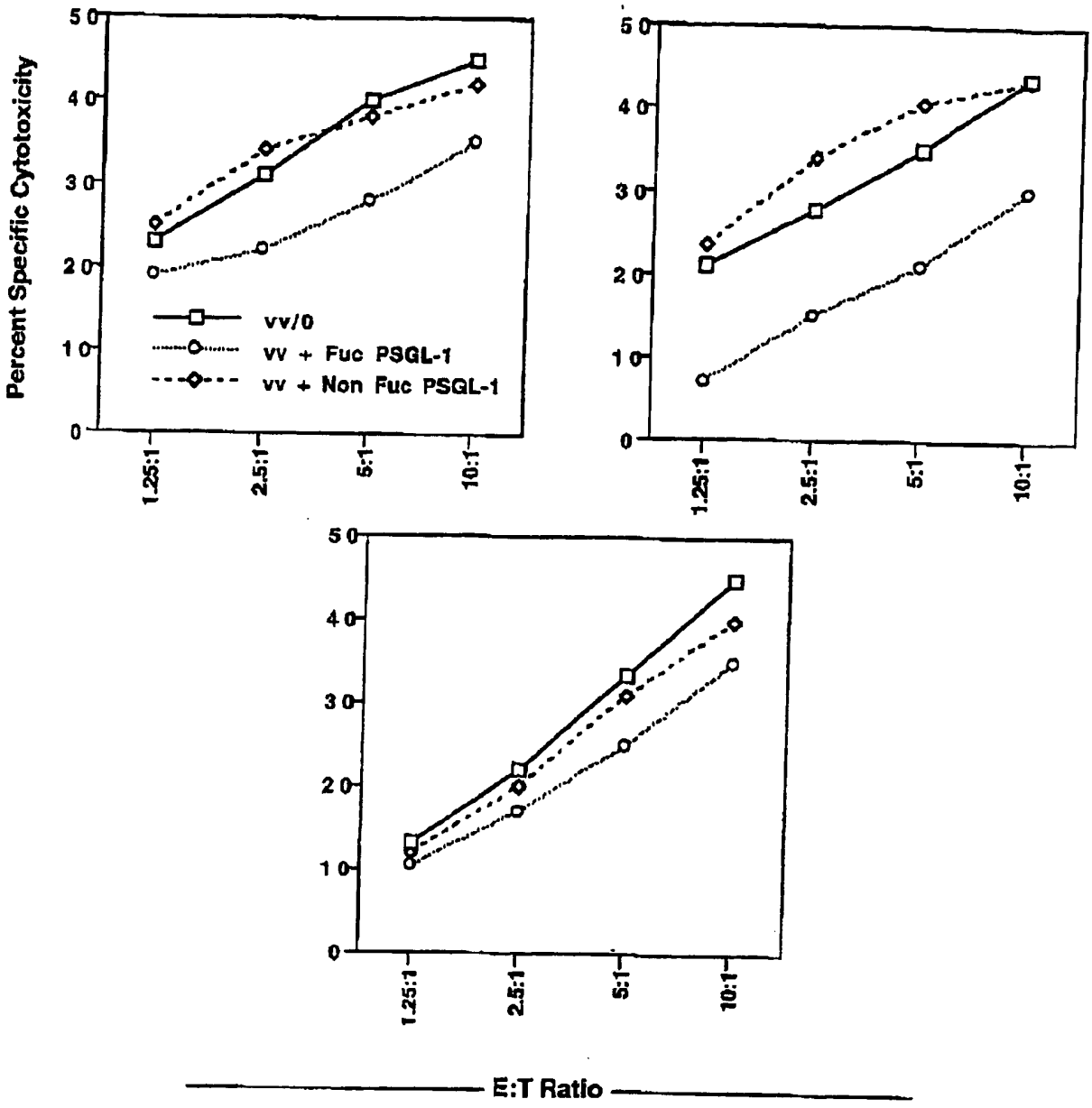

vv/0
vv + 2PH-1
vv+Mel-14
Percent Specific Cytotoxicity
0
20
40
60
80
1.25:1
2.5:1
5:1
10:1
E:T Ratio

Fig 7B


Percent Specific Cytotoxicity
50
40
30
20
10
0
vv/0
vv + Fuc PSGL-1
vv + Non Fuc PSGL-1
1.25:1
2.5:1
5:1
10:1
E:T Ratio

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/25501

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :A61K 38/16, 38/17
US Cl. :514/2, 8, 885
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/2, 8, 885

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DIALOG, BIOSIS, CA, EMBASE, MEDLINE, USPAT
search terms: psgl, graft, transplant, cytototox?, T cell, inhibit?, suppress?, antagoni?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,827,817 A (LARSEN ET AL.) 27 October 1998, see entire document, including column 15, paragraph 2.	1-4
X	US 5,659,018 A (BERNDT ET AL.) 19 August 1997, see entire document, including column 7, paragraph 4.	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
17 FEBRUARY 2000	07 MAR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PHILLIP GAMBEL Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/25501

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/25501

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I. Claims 1-4, drawn to methods of inhibiting cytotoxic T cell differentiation with PSGL antagonists.

Group II. Claims 5-8, drawn to methods of ameliorating autoimmune conditions with PSGL antagonists.

Group III. Claims 9-12, drawn to methods of ameliorating allergic reactions with PSGL antagonists.

Group IV. Claims 13-16, drawn to methods of ameliorating asthma with PSGL antagonists.

These methods require different ingredients, process and endpoints and encompass conditions or pathologies that differ in etiologies; therefore, they are distinct.

This application contains claims directed to more than one species of the generic inventions. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species of Groups I/II/III/IV are as follows:

a) PSGL
b) PSGL-specific antibody,
c) sLex-specific antibody,
d) sulfated tyrosine-specific antibody,
e) sLex,
f) mimetics which inhibit sLex, or
g) small molecule inhibitors.

These molecules differ in their structures, expression and modes of action; therefore they are distinct.

The inventions listed as Groups I/II/III/IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical feature of Group I is considered to be methods of inhibiting cytotoxic T cell differentiation with a PSGL antagonist. Berndt et al. teach mocarhagin which is a PSGL antagonist to treat various conditions, including inhibiting transplant rejection (see entire document, including column 7, paragraph 4). Larsen et al. teach the use of PSGL as a PSGL antagonist to treat various conditions, including inhibiting transplant rejection (see entire document, including column 15, paragraph 2). Therefore, Group I does not provide for a special technical feature over the prior art. Accordingly, Groups I-IV and the corresponding species are not so linked by the same or a corresponding special technical feature over the prior art.